Occurrence and Potential Health Effects of Antibiotic Resistant and Pathogenic Enteric Bacteria on Swine Animal Agriculture and Row Crop Farms in Farmers and their Neighbors

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Abstract

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(Under the Direction of Mark D. Sobsey)

Antibiotic resistant (AR) and pathogenic enteric bacteria are of human health concern. Antibiotic use in high density animal agriculture (CAFOs) is a potential source of human exposure to these bacteria. This pilot study was intended to assess impacts of CAFOs on human pathogens (*Salmonella*) and AR enteric bacteria (*E. coli* and *Enterococcus*) on environmental waters and people living near or working on these facilities. Eleven swine CAFOs were compared with six row crop farms for occurrence and frequency of AR bacteria in ground and surface water. Fecal samples were collected from 87 people associated with both farm types, to assess risk of AR enteric bacteria carriage. High concentrations and frequencies of AR *E. coli, Enterococcus*, and *Salmonella* were found in swine wastes; they were also found in surface waters but at lower concentrations. *E. coli* or *Enterococcus* concentrations were not significantly different when comparing upstream and downstream samples within farm types. However, *Salmonella* concentrations were significantly higher in surface water downstream of CAFOs than upstream. Bacteria concentrations of downstream surface waters were not significantly different between CAFOs and row crop farms. Risk of AR carriage was higher in people associated with CAFOs (RR= 1.42 [95% CI =1.17 1.72]) but the proportion of human

isolates with multiple AR was higher among those people associated with row crop farms. As concentrations of bacteria in waters of both farms types were not statistically different and phenotypic links between the bacteria found in animal wastes, water and people could not be established, the AR bacteria in human stool samples could not be attributed to the farms. This study found high frequencies of AR bacteria on CAFOs and that people associated with CAFOs had higher risk of carriage of AR bacteria than people associated with row crop farms. However, those associated with row crop farms had bacteria with more resistance traits. Further analysis on multiple CAFOs is necessary to increase statistical power and to establish links, if any, between AR bacteria found on farms and in people to conclusively assess impacts of swine agriculture on human health effects associated with AR and pathogenic enteric bacteria.

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Table of Contents

List of Tables

List of Figures

Chapter 1 – Introduction

With the advent of antibiotics in the 1940s, it was believed that infectious diseases were on the decline and would soon be greatly reduced if not eradicated. Antibiotics were considered the "silver bullet" that would remedy the scourge of numerous infectious disease agents and their illnesses caused by pathogenic bacteria that had plagued people worldwide. While these drugs did have an enormous beneficial effect, intrinsic resistance and development of extrinsic resistance has required the development of newer antibiotics and compromised the effectiveness of many antibiotics. Studies have consistently demonstrated that persons infected with antibiotic resistant pathogens require longer hospitalizations at a higher cost, and in many cases have increased morbidity and mortality.

Bacteria have the ability to survive extreme conditions. Specialized bacteria have evolved to live in the depths of the oceans free of any oxygen and light; they have evolved to live in hot springs where there are extremely high temperatures. Some bacteria require oxygen while others need anoxic conditions; some need neutral pH while others need acidic or basic conditions. The range of different environmental conditions to which bacteria have adapted is extensive. While not all of the mechanisms of survival and continued growth are completely understood, one thing is clear: bacteria will find a way to survive and often proliferate in the environmental conditions to which they are subjected.

Bacteria have the ability, through mutation and acquisition of genetic material to survive and proliferate better in a changing environment. There are two major ways by which bacteria acquire genes: mutation and acquisition of genes from other bacteria. In either case, the genes that promote survival or can help the bacterium out-compete other organisms are maintained and passed on to their progeny while those genes that that do not are either lost or not expressed.

The introduction of antibiotics and their widespread use for therapeutic, and non-therapeutic (e.g., enhanced growth of farm animals) purposes created another environmental condition to which bacteria were forced to adapt in order to survive. Many bacteria have acquired genes that enabled resistance to the various drugs. Today, we are again facing a situation similar to that of the pre-antibiotic era: some cases for which bacterial infections and diseases have no effective treatment.

As the problem or antibiotic resistant infections has emerged and become pervasive, the ways in which antibiotic use can be reduced has been explored. Antibiotics are used in human and in veterinary health for treatment as well as preventative purposes. In addition, antibiotics have been used for growth promotion purposes in food animal agriculture and aquaculture. Campaigns within the United States as well as other countries and regions have begun to implement the prudent use of antibiotics. This includes educating doctors as well as the public on better practices for the use of antibiotics (CDC, http://www.cdc.gov/drugresistance/community/anitbiotic-resistance.htm). To encourage prescribing and/or taking antibiotics when an individual has a bacterial infection (and not a viral infection); and when prescribing medication, being sure that the entire dose prescribed is taken, not just until the patient is feeling

better. Additionally, efforts have been made to reduce or eliminate the use of antibiotics at sub-therapeutic levels in animal agriculture.

Efforts have been made to reduce the use of antibiotics in food animal production. Regulations have been established that prohibit the use of antibiotics several weeks prior to the animals' slaughter. Furthermore, there have been bans on animal agriculture use of certain antibiotics or certain classes of antibiotics that may increase resistance to certain drugs that are essential in human medicine. For many stakeholders, however, these reductions in use are not enough and some would like a ban on all antibiotic use in food animals at sub-therapeutic levels.

There are some important reasons for which this use of antibiotics is considered necessary by its advocates. The majority of food animal production in the United States and many other countries is conducted on very large scale farms known as Contained (Confined) Animal Feeding Operations (CAFOs). In these facilities hundreds to thousands of animals are housed in a single facility. In these high animal density conditions, it is essential to maintain animal health as well as ensure animal growth at approximately the same rates and with high feed (nutrient-to-biomass conversion) efficiency. The use of antibiotics at sub-therapeutic doses aids in achieving these goals. Eliminating the sub-therapeutic use of antibiotics for these purposes could potentially result in higher incidence of illnesses among herds, large reductions in herd size and higher costs of production. All of these effects could lead to much higher costs to the consumers of these food animals. In addition, a ban in this country but not worldwide, could result in the exportation of this industry to other countries. If this were to occur, there could be fewer regulations on the production of food animals in other countries that

would export their products to the United States, resulting in less safe products for the consumer.

Given the potential for serious negative effects resulting from a ban on antibiotics in animal agriculture, it is important to clearly understand and assess the risks of antibiotic resistant bacteria originating in animal agriculture facilities. To do so, it is first necessary to determine if in fact antibiotic resistant bacteria are present in food animals and in their waste streams, if those bacteria are entering the environment, are present in the animal products sold to consumers, and if people are getting otherwise exposed to and acquiring antibiotic resistant bacteria that originate on farms.

To date, the majority of research on antibiotic resistance and food animals has focused on the risk to consumers of animal food products. While there have been a few studies that have examined the effects of antibiotic resistance on animal farm workers, there has been less research on the environmental impacts of antibiotic resistance or the potential for environmental exposures to and health effects of these bacteria on people who live near these large animal facilities. This research is intended to address some these issues by investigating antibiotic resistant bacteria in swine wastes, in the waters of swine farms and for reference, in waters of non-animal agriculture (row crop farms) and in people working on or living near both types of farms.

Chapter 2 – Objectives and Research Question

Objective

This study is designed to determine if human exposure to animal-related, specifically swine-related, agriculture environments results in an increased risk of acquiring or carrying antibiotic resistant bacteria or *Salmonella* and the illness salmonellosis when compared to those exposed to non-animal agriculture, specifically row crop farm environments.

Research Question or Purpose

Exposure to antibiotic resistant bacteria and bacterial pathogens in food animals is of growing concern. To date, the majority of research has been focused on the food borne route of exposure. Little research has been done with regard to environmental water exposures to pathogenic and antibiotic resistant bacteria from food animals and their agricultural production environment. As a result, human health risks posed by environmental exposures to antibiotic resistant bacteria, and pathogens, from food animal facilities are uncertain. This research is intended to 1) quantify enteric bacteria, including fecal indicator species *E. coli* and *Enterococci* sp. and the pathogen *Salmonella,* present in animal waste on swine CAFOs and in ambient waters associated with these facilities compared to water associated with row crop farms; 2) analyze human fecal samples from

people working on or living near swine agriculture and row crop farms for antibiotic resistant bacteria and *Salmonella*; 3) Characterize the bacteria found in the environment (swine wastes and farm-related environmental waters) and human fecal specimens of people working on or living near study farms for their phenotypic antibiotic resistance and determine if there are links between the bacteria found in these environmental samples and those isolated from humans working on or living near these farms; and 4) assess the potential human health risks of antibiotic resistant enteric bacteria and *Salmonella* pathogens from swine facilities. Environmental samples are to be obtained and analyzed seasonally for a year and human fecal samples are requested to be submitted monthly and during episodes of diarrhea over the course of a year for isolation and characterization of antibiotic resistant enteric bacteria and *Salmonella*. Bacteria from environmental samples and humans will be compared biochemically and phenotypically to determine if they are likely to be the same and have common origins or sources. The isolation rates and characteristics of bacteria from people associated with swine agriculture and row crop farms will be statistically compared to determine if they are the same and pose similar risks or if one of the two kinds of farms poses significantly greater risks than the other.

Chapter 3 – Background and Experimental Approach

Background

Enteric pathogens, bacterial indicators, and routes of transmission and exposure

Microorganisms, including bacteria, are not only ubiquitous in the environment they serve an important role in all ecosystems. Whether it be in soils, water, are or in or on flora and fauna, bacteria acts to maintain the normal cycles of life. However, in the context of public health, the role of microorganisms is seen in an entirely different light. It is not just the bacteria's role in life cycles that is of concern but rather how these bacteria impact humans and human health.

Human pathogens are of particular concern, in that these organisms have demonstrated their capacity to cause illness. While much of the transmission of pathogens occurs via person to person, there are other potential routes, including transmission via environmental sources and vehicles. Environmental contamination from fecal waste such as human sewage is of major concern due to the likelihood that human fecal matter contains human pathogens. These pathogens include, but are not limited to enteric viruses such as hepatitis A virus, enteroviruses and noroviruses; bacteria such as *E. coli, Salmonella* spp., *Campylobacter* spp.; protozoa parasites such as *Giardia lamblia, Cryptosporidium parvum* and helminth parasites such as *Ascaris* ova.

Once pathogens are introduced into the environment they have the potential to cause serious harm if humans are exposed to high enough concentrations. This is further compounded by the fact that many of these organisms have relatively low infectious doses (high probabilities of infection from exposure to low numbers of microbes). Therefore even a relatively small amount of waste harboring pathogens and introduced into the environment has the potential to cause illness in exposed humans. Additionally, because the concentrations of these organisms in the environment are likely to be low, it is often difficult to identify their presence. Furthermore, performing analyses to identify and quantify these pathogens in the environment is often time consuming, costly and inefficient. These factors make it difficult to monitor the environment for pathogen contamination.

An alternative is to find other methods by which information regarding fecal contamination and its source can be gathered. One commonly used technique is to detect and quantify indicator microbes. A good indicator microbe must have traits similar to the pathogens that they are intended to represent or predict. For example, they must be able to survive in the environment for at least as long as the pathogens of concern. The indicator must provide at least some information with regard to the source of the contamination. For example using a bacterial species that is found only in human gut flora is more useful in identifying a human source of contamination that one that is present in all mammals. And finally, a good indicator is abundant compared to the pathogens and can be cultured or otherwise detected from the environmental samples efficiently, quickly and inexpensively (WHO, 2001). Once good microbial indicators are

identified, it is then possible to test areas in which either fecal contamination is likely, or in places where there is an increased concern for human contact.

There are different routes of transmission for these enteric pathogens and therefore all of the potential routes should be considered when identifying where and how and individual may be exposed. Although this study focuses on waterborne exposures, it is also possible that other exposure routes, such as direct and indirect animal and human contact, airborne exposure, and contact with fomites, soil and vegetation could also be exposure sources. It is beyond the scope of this study to examine all of these possible exposure routes to enteric pathogens and antimicrobial resistant bacteria of animal agriculture origin. However, the possibility that these other routes are responsible for human exposures must be considered somehow when water is being investigated as the exposure vehicle.

One of the primary routes of transmission for enteric pathogens is via ingestion. For this reason, it is important to be sure that sources of drinking water are essentially pathogen free (no detectable pathogens present). This includes both public water sources such as piped systems originating from surface water (e.g., reservoirs) as well as well (ground) water. In addition to drinking water, people come in contact with water for recreational purposes. These include activities involving not only indirect exposures, such as fishing, but also direct exposures with ingestion such as swimming and bathing. When engaging in any of these activities there is a possibility that some of this water may be ingested. For this reason it is important that these water sources are managed and monitored for levels of fecal contamination.

While much of the emphasis in current research, management and associated monitoring is on sources of human fecal contamination, other sources of fecal contamination cannot be discounted. More and more information is becoming available regarding the risks to human health from zoonotic diseases (human infections from vertebrate animals). While not all animal pathogens may cause human disease, there are many zoonotic pathogens that have been identified (>300) such as *Salmonella* sp. commonly associated with poultry, *Campylobacter jejuni* associated with poultry and sheep, and *Cryptosporidium* and *Giardia* associated with cattle as protozoan pathogens. More and more zoonotic pathogens are being found or are emerging that do affect human health, including viruses such as SARS Coronavirus and avian influenza virus type H5N1 (WHO, 2004). Furthermore, while some of the bacteria found in animal fecal matter may not be frank human pathogens, they have the potential to transfer genetic traits such as antibiotic resistance to bacteria found in humans, including human pathogens and to environmental bacteria encountered by humans, which could also increase exposures and lead to possible risk to human health.

Antibiotic Resistance, its Impact on Public Health and Mechanisms of Acquisition

In the world today there is an increasing awareness of and concern about antibiotic resistance and its human health implications. When antibiotics were first discovered and used to prevent illness, it was thought that human infectious diseases would soon be greatly reduced if not eradicated. While the availability and widespread use of antibiotics was certainly a huge advance in the fight against infectious disease, other problems arose. Almost immediately after the introduction of the drugs, bacterial

strains that were resistant to them began to appear. Now, about 60 years on, we are faced with a situation approaching that of the pre-antibiotic era: cases in which bacterial infections arise that can not be treated or controlled to due antimicrobial resistance.

Bacteria have the capacity to change and genetically adapt to various environmental conditions. Random mutations within their chromosome that prove to be advantageous are selected for and can become "normal" within the population after a few generations. Additionally, bacteria have the ability to acquire genes from other organisms to enhance their survival. Three mechanisms by which bacteria can acquire new genetic information are transformation, conjugation, and transduction. Conjugation is responsible for the majority of bacterial genetic transfer in the environment (Davison, J., 1999). Plasmids can contain a variety of genes that be transferred to other bacteria by conjugation. Such transfer is not restricted to bacteria of the same species and can cross to other species and genera and these plasmids often carry genes that encode for resistance to one or more antibiotics (Aarestrup, F.M. and Wegener, H.C. 1999, Sunde, M., and Sorum, H, 2001, Gilmore, M.S., and Ferretti, J.J., 2003, Martinez-Martinez, L. et al., 1998).

This type of genetic transfer of antibiotic resistance genes has been detected in a variety of environments and includes a variety of different bacterial genera/species. In One case, multi-drug resistant and vancomycin resistant *S. aureus* was isolated from a foot ulcer. It was later discovered that the *S. aureus* bacteria had acquired its resistance genes from vancomycin resistant *E. faecalis* in the same patient (Brumfiel, G., 2002, Ferber, D., 2003). Other studies have found that this genetic transfer can occur among bacteria in the environment including those bacteria found in the soil, in water, animals

and humans (Nwosu, V., 2001, Gilmore M.S. and Ferretti, J. J., 2003, Klare, I. et al, 2003, Stine, O.C. et al, 2007, Bischoff, K.M. et al, 2005).

In the presence of antibiotics, the likelihood of conjugal transfer of plasmids encoding for antibiotic resistance genes is high. This type of transfer can easily occur in the intestine of humans and animals and is likely given the high concentration of bacteria in these environments, and has taken place with a variety of both gram negative and gram positive bacteria (Sunde, M. and Soren, H., 2001). Conjugal transfer of plasmids in the human intestine has been documented by plasmid analyses of clinical isolates during outbreaks in which the isolated bacteria were antibiotic resistant (Davison, J., 1999).

The implications of bacterial acquisition of antimicrobial resistance genes are profound. Bacteria exist everywhere and are required for life in general. Historically, the major concern has been for pathogenic bacteria and resulting illnesses. With the increasing presence of antibiotic resistant strains of bacteria in the environment and their ability to transfer genetic material to pathogenic organisms, the normal human flora, including those of the gut, become carriers that can turn susceptible pathogens into resistant pathogens and harmless commensal organisms into human health threats (Gilmore, M.S., and Ferretti, J.J., 2003).

Human studies show that individuals do not need to come in direct contact with antibiotics themselves to acquire resistant bacteria. Coming in contact with bacteria in the environment that carry the resistance genes is sufficient. In a study by Rahim, S., et al. (2003), it was seen that resistance to Linezolid, a synthetic antibiotic, could be conferred to patients with no direct exposure to the antibiotic but who stayed in the same hospital as those receiving treatment with the antibiotic. This study demonstrates that there is a

method of transfer for antibiotic resistant bacteria via fomites or other routes of exposure, not simply direct exposure to antibiotics.

While the study on Linezolid provides insight into the behavior of bacteria and transfer of antibiotic resistance among humans in general, it leaves questions with regard to the likelihood of transfer of these traits from non-human colonizing bacteria to those which colonize humans. For conjugation to take place and be effective, the bacteria must be in close enough contact at high enough concentrations for contact between them to be likely. Sorensen, T.L. et al. (2001) found that subjects who ingested both glycopeptide (e.g. vancomycin) and streptogamin resistant *Enterococci*, found in meat and meat products, were able to harbor and shed these bacteria for up to two weeks post ingestion. This study demonstrated the ability of bacteria found in food animals to not only survive the conditions of the human gastrointestinal tract but also colonize and multiply for up to two weeks. Two weeks may be enough time for bacteria to confer resistance genes to species that are endemic to humans or those that are human pathogens. These results demonstrate an environmental vehicle and human host exposure situation in which conjugation could potentially occur.

In response to the ability of bacteria to acquire and transfer antibiotic resistance genes, efforts have been made to identify the sources of and when possible limit the presence of antibiotics in the environment to try to prevent the selection of resistance genes. Limiting these exposures is difficult and in some cases impossible. Not only are some antibiotics present naturally in the environment, but the use of antibiotics for both human and animal health is extensive. The best option is to reduce usage whenever possible.

Worldwide there are attempts to reduce the amount of antibiotics used. Many policies were established to abolish or change practices that are thought to be major contributors to the problem. These include strict protocols for the use of antibiotics for treatment purposes (e.g. tuberculosis treatment regimens (WHO, 2003), and in veterinary practices the specific drugs that can be used and in what doses (van den Bogaard, A.E. 1999, Sainsbury, D.W., 1999). In the United States and Europe, the use of certain antibiotics has been banned as a result of growing concerns for the spread of resistance (Aarestrup, F.M., 2000). In 2005, the FDA succeeded in having Bayer remove an antibiotic used in animal agriculture that is in the fluoroquinone class, enrofloxacin (Baytril®), from the market (Kaufmann, M. *Washington Post*, 2005). Furthermore, reducing the use of other antibiotics in animal agriculture has become an issue. Many people feel that sub-therapeutic use of antibiotics in animal agriculture is non-essential and therefore should be banned, allowing antibiotics to be used in veterinary medicine only under strict prescription for a specific animal that is ill. Others feel that general use of antibiotics is an essential practice for economical food production.

Animal Agriculture and Antibiotic Usage and Potential Impact

Animal agriculture is a growing industry worldwide. In North Carolina alone (2002 statistics), there are about 9.9 million pigs and 10.6 million chickens (layers, 20 weeks old or older) (USDA, 2003). With the increasing human population, demand for animal products has increased, but the land available for production facilities has decreased. As a result, many facilities house thousands of animals at a single location called contained or confined animal feeding operations (CAFOs). With high

concentrations of animals at a single location, it is essential that facility operators maintain healthful facilities and healthy animals and use practices that will promote animal growth not only more quickly and with better feed efficiency but also at the same rate. Antibiotic supplements in animal feed are used to achieve these objectives. Antibiotics use at sub-therapeutic levels enables faster animal growth with less feed and at similar rates. Antibiotic use also helps to maintain the overall health of the animal cohort (Phillips, I. et al. 2004).

To understand the impact of the usage, in Europe, an estimated 1.6 million kg of antibiotics were used for growth promotion purposes in 1997, and about 5.5 million kg were used for human health purposes (Teuber, M., 2001); in the United States, it is estimated that 23 million kg of antibiotics are produced and that about 40% of that is used animal agriculture, the majority of which is used in sub-therapeutic doses (Esiobu, N. et al, 2002, Levy, S. 1998). Though animal growth promotion use has been practiced for several decades, it has recently come under more intense scrutiny due to the high concentration of animals, high quantities of animal wastes, such as manure, the better understanding of zoonotic pathogens and disease, and the public health concern of antibiotic resistance among the bacteria in the animals and their wastes.

While there have been many studies that have demonstrated that there are antibiotic resistant bacteria as well as antibiotic residues present in some animal agriculture facilities from the animal feces to the treatment systems such as lagoons (Wiggins, B.A., 1996, Chee-Sanford J.C., et al., 2001, Campagnolo, E.R., 2002, Donabedian S. et al 2003, Garcia-Migura, L. et al., 2005, Travis, R.M. et al., 2006, Jackson, C.R. et al , 2007, Martins de Costa, P., et al, 2007, and our laboratory(data

unpublished)), there have been fewer studies with regard to the impact on humans and human health. The majority of studies that have examined the impacts of antibiotic resistance on human health have focused on consumption of animal products that may contain antibiotic resistant bacteria. Evaluation of bacteria in consumer meat products has identified antibiotic resistant bacteria in these food products (Schroeder, C.M et al 2002, Messi, P. et al., 2006). Furthermore, some outbreak investigations have found evidence of a link between consumption of contaminated meat and illness. In 1998 an outbreak of an unusual strain multi-drug resistant *Salmonella enterica* serotype typhimurium occurred in which pork from a slaughterhouse was directly linked to cases of disease. Several people who ate the tainted meat became ill, as well as some that worked at the slaughterhouse at which the infected animals were killed. There was further illness from secondary transmission; however there was substantial evidence not only to identify the pork as the source of these bacteria but to trace it back to the slaughterhouse as well as the farm from which the infected pigs had come (Molbak, K. 1999).

In addition to those studies that have focused on the consumption of meat and other animal products, there have been a few studies that have examined the environmental exposures that may lead to acquisition of antibiotic resistant bacteria. Studies by Levy, S. (1978), van den Bogaard, A.E., et al, (2001 and 2002) and Aubry-Damon, H. et al. (2004) reported increased incidence of chicken and swine farm workers acquiring antibiotic resistant bacteria when working in animal agriculture facilities that use antibiotics. Those who come in direct contact with animal feces and those antibiotics

used in the facility are more likely to acquire resistant bacteria. However, information is lacking with regard to the risks to those living in close proximity to these farms.

There have been a limited number of studies that have examined the impact of antibiotic resistant bacteria originating from animal agriculture on the environment around the farm. Giggs, S.G. et al (2006) found resistant bacteria in the air as far as 150 meters downwind of a swine CAFO. This finding indicates that those who live in close proximity to this farm may have increased exposure to resistant bacteria. Chee-Sanford J.C., et al. (2001) found tetracycline resistance genes in ground water under two swine lagoons. The presence of these genes in the water may allow bacteria that are also present to become resistant. As many people who live in rural areas utilize ground water wells as their drinking source, and these well are often untreated, this could potentially lead to an increase in exposure to resistant bacteria. And finally, studies by Johnston, L., and Jaykus, L.A. (2004) and Senegelov, G. et al. have found that manure or manure slurry applied to fields or fields that have been spray irrigated with lagoon waste have an increase in resistant bacteria. These bacteria can survive in the soil as well as on produce. Often the spread or spraying of the manure or manure slurry is close to the animal facilities themselves. Therefore, people who live near these facilities may come in contact with these soils or each contaminated produce may have an increased risk of acquiring resistant bacteria.

With the increase awareness and concern of antibiotic resistance among human and animal bacteria, policy makers in the United States are charged with important decisions with regard to antibiotic use. Without adequate knowledge of actual impacts on human health and the environment, informed decisions can not be made. While many

people would like to abolish antibiotic use in animal agriculture, this could have serious ramifications to the economic well being and livelihood of farmers, their communities, the general public and the costs of food.

This study is intended to provide insight into the potential human health effects of antibiotic resistant bacteria and *Salmonella* in the environment by determining whether or not animal agriculture, specifically swine CAFOs, are sources of such contamination and human exposure.

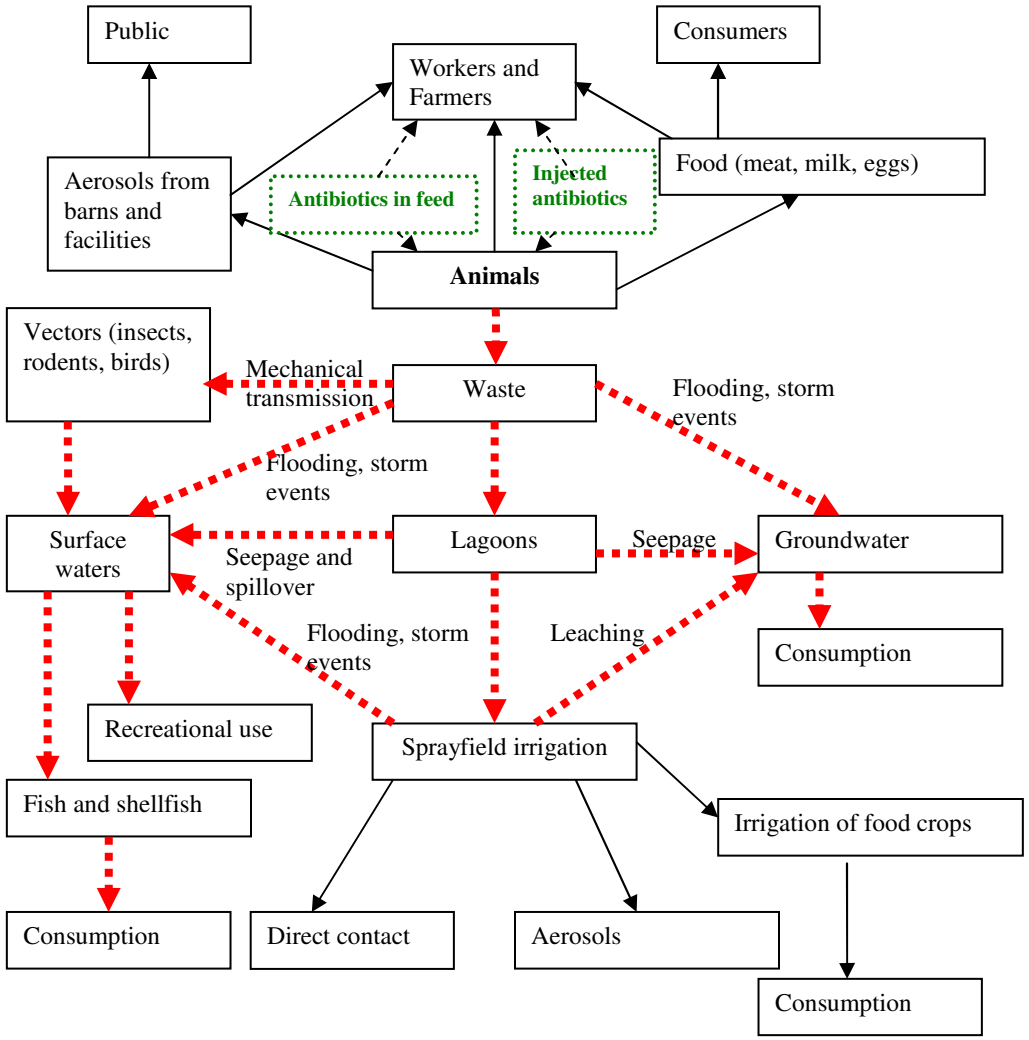
There are several pathways by which bacteria originating from CAFOs may enter the environment and result in human exposure. A schematic of some of the potential pathways of human exposure to bacteria originating in CAFOs is seen in Figure3.1; those pathways that are related to waterborne exposure are highlighted with the dashed arrows. Many of these pathways lead to consumption of contaminated products by consumers. While some of these products are products of the animals themselves, there are several routes of exposure by which other consumer products are contaminated. This includes contamination of produce. One route by which produce become contaminated is by contact with the untreated animal feces directly. This can occur by using animal manure for fertilizer, or by utilizing animal waste lagoon water for irrigation. An additional route by which the crops may be contaminated is via irrigation with contaminated canal or stream water. An example of this is the 2006 *E. coli* O157:H7 outbreak involving spinach. In this case, it is suspected that several cattle ranches upstream of the spinach farm contaminate the irrigation water that was later used on the spinach (Maki, D.G.,

2006). While this was not an instance for which the bacteria causing illness were resistant to antibiotics, similar exposure with resistant bacteria could occur.

Given the many ways by which people can be exposed to bacteria that originate in animal agriculture facilities, it is important to understand not only how one may become exposed by also to what they are exposed. Identifying the types of bacteria, particularly potential human pathogens as well as the concentrations of these bacteria is essential. Furthermore it is important to understand the different characteristics of these bacteria including their antibiotic resistance profiles.

Salmonella and multiple antibiotic resistant *E. coli* and Enterococci have been found to be present in swine, including those in NC (research in our laboratory – no yet published). Concentrations of multiple antibiotic resistant bacteria, including *Salmonella* are high in untreated swine wastes and there are still readily detectable levels of these bacteria in land applied swine lagoon liquid. Swine waste storage or typical treatments do not appear to appreciably reduce the extent of antibiotic resistance among bacteria remaining in waste residuals. This research is designed to further understand the extent to which these bacteria affect those who are associated with animal agriculture.

Figure 3.1: Schematic of potential sources of human exposure to antibiotic resistant bacteria originating from swine farms



(Adapted from Casanova and Sobsey, unpublished – submitted Aug 2005) (Difference in line type and source for antibiotics added)

Microbial Source Tracking

Microbial Source tracking has become an important tool in identifying sources of fecal contamination in the environment. Using different types of analyses and different target microbes, it is possible to identify "fingerprints" or other unique identifying characteristics of the bacterial isolates that help establish parentage and other links among

them that would not be possible with the more traditional phenotypic methods that detect only genus or species. There are different microbial source tracking methods that can be used to help identify potential sources of bacterial isolates. Each target microbe and analytical method has pros and cons, and the utility of the specific microbe and method varies depending on many factors. These include the parameters of the study such as its location and microbial sources, the number of isolates that need to be tested, the availability of a library of source isolates to which the environmental sample isolates can be compared and the technical capacity and funding available to conduct the analyses.

There are molecular and non-molecular methods for microbial source tracking. Some non-molecular methods include cultivation techniques that look for different gastrointestinal microbes present in different mammal species (US EPA, 2005). These techniques include analyzing fecal coliform/fecal streptococcus ratio; identifying the presence of certain bacteria that are abundant in animal colons such as *Bifidobacterium*, *Bacteroides, Eubacterium, Clostridium, Ruminococcus, Peptococcus, Peprostreotococcus* and *Fusobacterium* (which are common in the human intestine but rare in animals); and identification of F+ coliphage groups and particular human enteric viruses. In addition to cultivation techniques, other non molecular methods can be used, such as conducting immunological assays that identify immunoglobulin types and sources, carbon utilization analyses and examining antimicrobial resistance patterns within the cultivated microbes (Scott, T.M. et al. 2002, US EPA 2005).

Antimicrobial resistance analysis (ARA) also known as multiple antibiotic resistance (MAR) is becoming more popular as attempts are being made to understand the issues surrounding antimicrobial resistance and its sources. Furthermore, this

technique has been shown to be effective in determining the species-specific source of fecal contamination, including both domestic and wild animal and human sources (Scott, T.M. et al. 2002). One reason for the success of this method is the difference in the types and quantities of antibiotic used for human treatments and for animal animals (Stewart, J. et al., 2003). For this reason, the selective pressure among the bacterial species could differ, resulting in difference resistance patterns. In this method the patterns of resistance in an isolate from an environmental sample are compared to a library of isolates from known but different potential sources. This approach has been used with a variety of bacteria including *E. coli* and fecal streptococci such as Enterococcus species (Simpson, J.M. et al, 2002, Scott, T.M. et al. 2002 and US EPA, 2005).

In addition to the non molecular methods, there are several molecular methods that have proven to be effective in identifying the source fecal bacteria and viruses. These methods include ribotyping, repetitive element PCR (including BOX-PCR), Randomly Amplified Polymorphic DNA (RAPD) analysis, Amplified Fragment Length Polymorphism (AFLP analysis, Pulse Field Gel Electrophoresis (PFGE), Length Heterogeneity PCR and Terminal Restriction Length Polymorphism, Denaturing Gradient Gel Electrophoresis, F+RNA coliphage typing, and gene specific PCR (often at multiple loci and referred to as multi-locus PCR). Additionally, there are methods that analyze an entire microbial community such as identifying 16S rRNA gene clone libraries (gene specific PCR), using host-specific PCR or using host specific quantitative PCR (QPCR) also known and real time PCR (RT-PCR) (US EPA, 2005).

Many of these methods require a library of known bacterial strains and/or nucleotide sequences of specific genetic loci or alleles to which the environmental isolate

can be compared. Furthermore, the cost and utility of each method differs. PFGE has been successfully utilized in epidemiologic studies to determine relatedness of bacterial strains and is widely used for molecular epidemiological studies in healthcare facilities and tracking food borne outbreaks (Scott, T.M. et al. 2002). Multi-locus PCR has emerged as one of the most powerful methods for microbial source tracking because it examines the exact genetic codes of specific genes or alleles within isolates of the target microbes of interest from different sources (comparing those from known and unknown sources).

With all these methods there are pros and cons to their usage. One important factor to note however is that many of these techniques are relatively new and therefore more studies are needed to demonstrate their usefulness. Additionally, statistical studies into library size of bacteria isolates from different sources is required for conclusive data that would strengthen the power of the methods to draw reliable and statistically supported conclusions (Stewart, J. et al. 2003). It is important to evaluate each technique within the context of a study and location to determine the best approach to use.

Summary

With the high concentrations of food animals in the United States (and the world), there is a need to fully understand the potential risks to public health from animal waste. While some zoonotic pathogens have been identified, there are still many potential human health risks arising from animal waste. The phenomenon of antibiotic resistant bacteria is of increasing concern and therefore it is essential that studies be conducted to

assess if CAFOs are a source of antibiotic resistance in humans. Determining presence and risks from bacteria of CAFO origin to potentially exposed humans may require the use of a number of different analytical approaches. Microbiological analyses of various kinds, coupled with epidemiological analyses offer the potential to examine and characterize microbial resistance, pathogen (e.g., *Salmonella*) occurrence and also their potential human health risks.

This study will attempt to address this issue by examining the potential for antibiotic resistant bacteria originating in swine farms to enter environmental waters to which people may be exposed. In this study we intend to quantify and characterize the bacteria in both swine facilities and ambient waters surrounding these facilities; analyze human fecal samples for bacteria that can be phenotypically linked via MAR to swine facilities; and assess the potential human health risks of antibiotic resistant enteric bacteria and *Salmonella* pathogens from swine facilities. In doing so we will attempt to determine whether or not swine farms in North Carolina pose a human health risk with regard to acquisition of antibiotic resistance or *Salmonella* infection.

Experimental Approach

1. Analyze environmental samples taken from large animal agriculture facilities, specifically swine farms, and also row crop farms in Eastern North Carolina for the presence and properties of *Salmonella*, and of antimicrobial resistant *Enterococcus* sp. and *Escherichia coli*
2. Obtain and similarly characterize antimicrobial resistant enteric bacterial isolates from fecal samples of people working on or living near these farms and any additional referent group.
3. Use phenotypic methods to compare the properties of and establish links for the antimicrobial resistant bacteria and *Salmonell*a found in the human participants and those found in the environmental samples.
4. Assess the potential for workers on farms and community members around these facilities to acquire antimicrobial resistant bacteria and *Salmonella* from the farm as a point source by utilizing both epidemiologic/statistical methods
5. Use statistical methods to compare swine workers and neighbors to non animal agriculture participants to determine any statistical differences in their acquisition of antimicrobial resistant bacteria in terms of types and properties of resistant bacteria and magnitude of acquisition or presence of these bacteria

Chapter 4 – Environmental Analyses

To assess the impact of animal agriculture on the environment and people in the surrounding communities, it is important to identify and quantify bacteria of known or possible animal origin on the farms. Such bacteriological analyses facilitate an understanding of what bacteria of animal waste origin may be released into the surrounding environment, including ground and surface water.

This study focuses contaminated water as the route of exposure to bacteria originating from farms and possibly farm animal waste. Therefore, to understand the potential impacts of the farm wastes on ambient waters it was important to examine the environmental waters for fecal bacteria and determine their concentrations. Surface water samples were taken upstream and downstream of animal agriculture facilities and analyzed for enteric bacteria, specifically, *E. coli Enterococcus* sp. and *Salmonella* sp. In addition, up and downstream samples of non-animal agriculture facilities (row crop farms) were also collected and analyzed for these bacteria. These water samples from row crop farms were used as a controls or references for comparison purposes.

By collecting up and downstream samples, it was intended to determine what bacteria, and at what concentrations, were in the water prior to its passage through the farm and then compare them to the bacteria in the samples after passage through the farm. Should there be an increase in bacteria concentrations going from upstream to downstream, the difference would be the assumed bacterial contribution of the farm.

Having a control group of farms (row crops) allows for better understanding of fecal bacterial concentrations in water where there was presumed less impact of animal agriculture. Bacteria levels in waters of row crop farms provides information regarding the background levels of bacteria in surface waters in the region, as well as possible microbial impacts of other types of farming on the environment.

Temperature and other weather conditions can have an effect on the type of and the quantity of bacteria present and surviving in the environment. Therefore, each farm and the surrounding waters were sampled three times per year in order to represent cool, moderate and warm weather seasons, based on normal, annual climate cycles for eastern North Carolina. Each farm was sampled at least once in each of these seasons. It was then possible to compare the concentrations found on the farms and in the water, and determine if there were any seasonal effects. As weather can be unpredictable, temperature and relative humidity measurements were recorded to account for any potential unseasonable weather conditions at the time of sampling. Precipitation events within 24 hours prior to sampling were recorded. During the study there were rain and snow events, and all of them were considered "normal" with regard to quantity of rain. One sampling trip was one week after remnants of a hurricane that produced a lot of rain and flooded the sampling area. However, this event was not considered "abnormal" flooding. There were no storms that resulted in major flooding such as 20-, 50- or 100-year storms.

There are several questions that were examined in this study. First, are the fecal bacteria *E. coli*, Enterococci and *Salmonella* sp. present in animal waste in animal agriculture facilities and in what concentrations? Are the concentrations of these bacteria

in fresh feces including barn flush the same as those found in the waste treated by lagoons? Do the bacteria originating on the farm impact the environmental waters surrounding the farms, including ground water and surface water streams? Do apparent bacterial contributions to surface water of the animal agriculture facilities differ from those of row crop farms? Are there any seasonal differences in the bacterial concentrations in waste samples and environmental waters?

The null hypotheses for these questions are that there are no differences in concentrations based upon sampling site (up or downstream of the farm), no differences based upon farm type (animal agriculture or row crop) and no difference based upon season.

Materials and Methods

Farm Selection

Eleven swine farms in Eastern North Carolina were selected to participate in this study. They represent each type of swine farm, i.e. sow, nursery and finishing farms; Of the 11, 1 was a sow farm, 3 were nurseries and 7 were finishing farms (table 4.1). All but two of the facilities had a flush system for waste removal from the house; the others used a pit recharge system. Each of these farms had a well on the property. All but one farm grazed beef cattle in addition to the primary swine operation. These cattle were generally fields adjacent to the swine barns and/or lagoons but were separated from them by fences. There was no waste treatment of cow manure. The animals defecated freely throughout the pastures.

Six Row Crop farms were selected. The row crop farms were identified within the geographic areas of these swine farms to act as controls or reference farms in the study. In some cases these farms were neighboring farms, in others they were within the same zip code. As with the swine farms, the row crop farms had streams on or bordering them. In one case, a row crop farm was remote from swine farms, and outside of the zip code but geographically within the eastern portion of North Carolina and within the same county as a study animal agriculture facility. This farm was selected to ensure that control farms have minimal, if any, impact from animal agriculture facilities.

As water was examined as the primary environmental route of exposure for this study, each of these farms was in close proximity to a non-ephemeral body of water; these farms either had a stream running through or as a border of the property.

Field Sampling

Ground and surface waters on and around the farm sites were collected for analyses. In addition, animal waste and waste stream samples were collected from the swine farms. If any type animal waste was land applied within a month prior to our sampling of a row crop farm, soil was to be sampled on the row crop farms. There were no instances in which land application of manure or spray irrigation of row crop fields occurred in the one month prior to sampling, therefore sampling of soil was not conducted during the course of our study.

Surface water samples were collected both upstream and downstream of the farm. Surface water samples were collected as close to the farm as possible. However, in some cases there was no access to a stream on the farm border so the closest stream access was

used to obtain the sample. These sampling sites were within 0.25 to, at most, 0.5 miles from the farm boundary. On one row crop farm, in addition to a stream, there were also irrigation ponds from which samples were also collected. These irrigation ponds had no stream inlets and therefore relied on precipitation for recharging. There were no animals grazing in the areas around the ponds however, they were not fenced either. Therefore, it was possible that wild animals including birds had access to these ponds as well as neighborhood pets such as dogs. Both birds and dogs were observed near at least one of these ponds during sampling.

Surface water samples were collected using a 12 foot telescoping pole with a sterile bottle attached to the end. Four to five "grab samples" were taken to fill a 4 liter bottle. Effort was made to reach toward the middle of the stream however, there were limits with the length of the pole as well as the environment at the sample site. In some cases the stream was very narrow and/or shallow and in these cases the widest and deepest source was selected to collect the sample. Once the sample was collected, its temperature was taken and the sample was placed in an iced cooler for transport back to the laboratory.

The telescoping pole was disinfected with 70% ethanol after each sample was collected.

Ground water samples were only collected on swine farms as no wells existed on any of the row crop farms. The wells on the swine farms were predominantly present to provide drinking water to the animals and assist with barn flush. All of the wells had piping that led to a tap (similar to those to which a hose could be attached) where water could be obtained. These taps were found inside the barn, on the outer wall of the barns

or, in one case, in a building about 20 yards from the barns but on the farm. In each case, the water was allowed to flow for approximately 30 seconds before the sample was collected in an effort to flush out potential contamination from the appurtenance or the water it held. Once the sample was collected, its temperature was measured and it was stored in an iced cooler for transport back to the laboratory.

The study was conducted over a two year period to enable each farm to be sampled three times in a calendar year. This was done to account for seasonal differences in conditions, especially temperature, which could influence the presence of bacteria: cool (November – March), moderate (October and March-May) and warm (June – September) seasons were delineated for sampling. There were four farms however, that were sampled a fourth time, repeating the warm season sampling period. This was done to account for logistical considerations regarding the human participant portion of this study. The goal was to have seasonal sampling during all periods when human samples were collected.

At the time of each sampling ambient air temperature and relative humidity were measured at each sample collection site. This was done to document prevailing seasonal conditions and account for any variations in normal conditions for that season. Additionally, weather conditions on the day of as well as the day before sampling, including precipitation events, were recorded.

GPS coordinates at each farm and at each sampling site were also recorded to assist in mapping and visualizing farm locations, proximities and exposures.

Environmental Sample Processing

Environmental samples were analyzed for *Escherichia coli*, *Enterococci* sp. and *Salmonella* sp. using quantal methods specific to each analyte and Most Probably Number (MPN) concentrations were calculated. Initial enrichment isolation was followed by streaking onto appropriate selective agar media for further confirmation. Several isolated colonies of each analyte from each sample were selected and archived, by being placed in Tryptic Soy Broth (TSB) with 25% glycerol and stored at -80ºC for further analyses. These analyses included biochemical identification to confirm the genus or species of each isolate and antibiotic resistance testing for all biochemically confirmed isolates.

E. coli were analyzed utilizing the Colilert™ system by IDEXX, which can quantify both fecal coliforms and *E. coli* when incubated at an elevated temperature. Samples were analyzed using quantitrays and the Colilert medium, with incubation for 24 hours; the first 3 hours at 37 ºC then the remainder at 44.5 ºC (the elevated temperature for fecal coliforms). Positive wells were counted (yellow color for fecal coliforms and blue fluorescence under long wavelength UV light for *E. coli*) and the MPN is determined using a table provided by IDEXX. Aliquots of 10µl from several *E. coli*-positive wells per sample were removed aseptically, placed on EC agar with MUG, over which was a 0.45µm filter, and streaked to isolate colonies. The plates were incubated for 24 hours at 44.5 ºC, and colonies that fluoresce blue under UV light were selected for archiving and further characterization.

Enterococcus sp. were analyzed using the Enterolert™ system by IDEXX. Samples are added to quantitrays with the Enterolert medium and incubated for 24 hours

at 41 ºC. Wells that fluoresce were scored positive for *Enterococcus*, tallied and quantified using the MPN table prepared by IDEXX. Then, 10µl aliquots from positive wells are streaked on Bile Esculin Azide agar plates that were incubated at 37 ºC for 24 hours. Brownish black colonies with a halo indicative of *Enterococcus* are selected for archiving and further characterization.

Salmonella sp. were analyzed using the 3-volume x 3-replicate per volume broth enrichment-colony isolation MPN method for water sample volumes of 900ml, 90 ml and 9 ml; samples that were likely to have higher *Salmonella* concentrations are 10-fold serially diluted.

Samples were pre-enriched in Buffered Peptone Water, incubating for18-24 hours at 37 ºC. The larger water sample volumes (900, 90, 9) were added to 100, 10 and 1 ml of 10X Buffered Peptone Water, respectively; 10ml/10g lagoon samples were added to 10mls of 2X Buffered Peptone Water; and the 1 ml volumes of undiluted or serial dilutions of samples were added to 1X buffered Peptone Water. In each instance, the final concentration of the pre-enrichment medium was a 1X solution.

After the 24 hour incubation, 100µl of pre-enrichment culture was transferred into 10ml of Rappaport- Vassiliadis broth, and incubated at 41 ºC for 24 hours for enrichment. From the enrichment cultures, approximately 10 µl were streaked onto Salmonella/Shigella agar using a sterile loop, and the plates were incubated at 37 ºC for 24 hours. Black colonies with a clear halo were counted as presumptive positive and several were selected for archiving. Salmonella MPNs were computed using the Thomas equation or standard 3 volume-3 dilution MPN tables.

Biochemical Identification of *E. coli* and *Salmonella* sp. was done using Enterotubes™, while Enterococci were biochemically identified using APi20strep™ strips. In some cases these strips yielded inconclusive results for which Enterococcus sp. were one or more of the options. When this occurred, the isolate was streaked onto TSA and incubated at 45 ºC; those that had growth were then re-streaked onto TSA with 6.5% NaCl to score for growth at this elevated NaCl concentration. These additional phenotypic analyses were chosen based on conditions under which Enterococcus could grow and the other possible species could not. Those bacteria that grew under both conditions (45ºC and 6.5% NaCl) were considered to be Enterococcus.

In those instances in which the biochemical tests (either the Enterotubes or Api20 strep strips) did not provide identification at least at the genus level, theses isolates were considered not to be the target organisms and no further analyses were conducted. For the Enterotube analyses, non-identification was scored when the code generated by the testing the organism was not identified in the codebook associated with this test. For the Api20strep strips, organism identification is based upon a probability that the organism in fact the one mentioned. For decision making purposes, any identification that had less than 95% certainty of Enterococcus was either further tested as described above, or concluded to not be Enterococcus. Furthermore, associated with the test kit identification were statements of likelihood of the genus, such as "good to the genus level" or "low species discrimination." These statements were considered in the final identification process.

MPN and Statistical Quantification – All bacterial concentrations were estimated using Most Probable Number (MPN) methods. These methods do not provide an actual

count of the bacteria present but instead an estimation of bacterial density or concentration based on a maximum likelihood that a certain quantity of bacteria is present based on the amount of sample analyzed and the number of sample volumes that score positive or remain negative of the total volume analyzed. In this study the estimation of MPN was based on the Thomas Equation, which is not an exact estimate but a reasonable approximation that is useful to use when the number of sample volumes and replicates of them are non-standard, thereby precluding use of standard MPN tables (Equation 4.1), ((FDA-BAM, 2001)). This equation was adjusted with a constant multiplier term to have data reported per 100 ml rather than per gram of sample as in the original equation (equation 4.2).

$$\mathbf{MPN/g = P/[(N*T)^{(1/2)}]} \qquad \textbf{equation 4-1}$$

Where:
P is the number of positive results,
T is the total grams in the sample in the selected dilutions
N is the grams of sample in negative tubes of the selected dilutions
(FDA-BAM, 2001)

$$\mathbf{MPN/100ml = P*100/[(N*T)^{(1/2)}]} \qquad \textbf{equation 4-2}$$

For each of these estimations an associated 95% confidence interval (equation 4-3), or the range in which the true concentration will be 95% of the time, that can be calculated by determining the standard error of the $\log_{10}$(MPN).

$$\textbf{Upper/lower 95\% CI} = \mathbf{Log_{10}(mpn) +/- 1.96*standard\ error} \qquad \textbf{equation 4-3}$$

For the Quantitray™ system (by which all of the *E. coli* and Enterococci sp. were quantified), IDEXX® has created its own MPN table based on Maximum Likelihood

equations to generate an MPN and its confidence limits for each combination of numbers of large and small positive wells. For these analyses this table was used to establish both the MPN as well as the 95% CI. Furthermore, these numbers were verified using the MPN generator program also provided by IDEXX®.

Farm Descriptions

Animal Agriculture Facility Descriptions

All participating swine farms were owned by independent growers. Each contracts with one of the larger pork producers in the region; the grower supplies the building, infrastructure and care of the animals while the larger companies (integrators) provide the animals and the feed. Although management practices are similar, animal maintenance and health care practices are dependent upon type of farm (sow, nursery, finisher) and may vary somewhat among integrators.

As a condition of the study, all farms in our study had non-ephemeral surface water flowing through or as a border to the farm. The type of surface water varied including streams, spring fed creeks and year round irrigation canals. The type of waste treatment systems for the swine farms was similar in that all used an anaerobic lagoon system with spray field irrigation for waste treatment and disposal. There were some variations by farm with regard to the number of lagoons present on the farm and in some cases there was a secondary lagoon for further treatment and/or storage of the waste. All but one (site 4) of the swine farms had cattle grazing on the farm. Other features by which the farms varied included: number of animals on the farm itself, the number of swine houses, and growth stage of the animal (e.g. sow, nursery, or finishing farm); Overall farm description are explained in detail below and summarized in table 4. 1.

Table 4.1 a&b: Summary Description of Study Farms

a. Swine Farm Type Breakdown

County	Site #	Type of Farm	# of Barns	# of Head Swine	# of Head Cattle	Waste Removal
Gates	12	Sow	11	4800 sows/ 30 boars	250	Pitt Recharge
Greene	6	Nursery	1	2800	25	Flush
Greene	1	Nursery	2	5600	20	Flush
Greene	5	Nursery	2	5600	30	Flush
Franklin	7	Finishing	10	7200	40	Flush
Jones	4	Finishing	8	7200	n/a	Pitt Recharge
Greene	11	Finishing	6	7444	65	Flush
Greene	9	Finishing	3	3672	65	Flush
Greene	10	Finishing	3	3672	65	Flush
Greene	2	Finishing	4	4896	65	Flush
Greene	3	Finishing	4	4896	65	Flush

b. Row Crop Farm Breakdown

County	Site	Crops grown	Proximity to CAFO
Gates	F	Corn, Beans	Upstream of study CAFO; no other CAFOS in the area
Franklin	D	Tobacco	No CAFOs within at least 1 mile
Greene/Lenoir	A	Cotton, corn	Adjacent to 2 study farms; downstream of one upstream of the second
Greene	C	Corn??	Downstream sampling site within 0.25 miles of non-study CAFO
Lenoir	B	Corn, beans	No CAFOs near sampling sites
Pitt	E	Corn, beans	Small CAFO upstream of farm

Site 1– This farm is a nursery facility located in Greene County. It consists of one house with single lagoon for waste treatment. The barn operates with a flush system by which recycled lagoon water flushed waste from the house into the lagoon multiple times per day. The animals are housed at this facility for approximately six weeks (up to about 40 lbs.- approximately 8-10 wks of age) and then sent to a finishing facility. The barn houses up to 2800 pigs at a time. In addition to the swine, cattle are grazed at this facility. Approximately 20 head of cattle are grazed in this area from March through October in the fields surrounding the farm, however the area directly surrounding the houses or the lagoon is fenced to prevent the cattle from grazing too closely.

Injectable antibiotics were rarely used at this facility, however, therapeutic doses of antibiotics may have been administered to the animals via their drinking water. These include tetracycline, chlortetracycline, sulfamethoxazone bisulfate and penicillin. Sub-therapeutic antibiotics were also administered constantly through the feed; the antibiotics used were not disclosed to the grower. As with finishing farms, there was an increase in vaccine usage which has decreased the need for antibiotics.

Site 2: This facility is located in Greene County and consists of four barns with a single lagoon for waste treatment. It operates with a flush waste removal system. This farm is a finishing facility at which the animals are housed from about 40lbs (8 to10 weeks of age) until market size (250 – 265 lbs); this generally takes 15 to 20 weeks. The farm houses up to 4896 pigs at a time. It also has about 65 head of cattle that graze in a fenced field approximately 50 yards from the swine houses and lagoon.

After eight weeks on the farm, antibiotics were no longer used on pigs for any reason. Prior to eight weeks, injectable penicillin was used to treat ill or injured pigs at

therapeutic doses. Occasionally, sub- therapeutic doses of chlortetracycline were given via feed, however, there was an increased usage of vaccines which has greatly reduced the need for antibiotic usage overall even at the sub-therapeutic levels. The antibiotic usage described here was similar to that of Sites 3, 9, 10 and 11.

Site 3: This is a finishing facility located in Greene County. This facility has four houses and two primary treatment lagoons. One lagoon receives waste flushed from three of the houses while the second lagoon receives waste from the fourth house. It utilizes a flush system for waste removal and houses up to 4896 pigs. About 50 yards from the swine houses, 65 cattle graze in a fenced field.

This farm had the same antibiotic usage as Site 2.

Site 4: This is a finishing facility in Jones County with eight houses that contain up to 7200 animals at a given time. The eight houses are flushed into a single lagoon using a pit recharge system in which the house floors are at least partially slatted and the houses are flushed approximately once per week.

Antibiotics were used at this facility mostly at sub-therapeutic levels through the feed. Therapeutic doses were given to the animals via drinking water. The antibiotics used at this facility were not disclosed to the farmer.

Site 5: For the purposes of this project this Greene County site is considered one study site. However, it consists of two separate farms that are approximately 300 yards apart. Each is a nursery facility that has one house with a flush system which discharges into a lagoon. Each farm houses up to 2800 pigs. About 30 cattle are grazed year round in the fields surrounding the two farms; however, fences prevented grazing in the immediate area around the houses and lagoons.

Antibiotics were used in both therapeutic and sub-therapeutic doses as in Site 1. The identities of the antibiotics were not revealed to the study team.

Site 6: Similar to Site 5, this Greene County nursery site is considered one study site but actually consists of two farms. In this case the two houses are approximately 200 yards apart with about 25 head of cattle grazing in the middle area. The cattle are only grazed at this facility from March through October. Each farm consists of one house that holds up to 2800 pigs and each operates a flush system that discharges into a single lagoon serving both farms.

Antibiotics were used in both therapeutic and sub-therapeutic doses as at Site 1.

Site7: This is a finishing facility located in Franklin County. It consists of ten houses and three lagoons. Generally, the lagoons operate in series as units for primary, secondary and tertiary treatment of the waste. However, the third lagoon is used for primary treatment of effluent from some of the barns, if the other lagoon levels are high. The waste is removed from the houses using a flush system. This facility houses up to 7200 pigs at a time. Cattle are grazed on a fenced field adjacent to the swine houses. It is in this field through which the creek flows. There are approximately 40 head of cattle grazing throughout the year. This facility used sub-therapeutic antibiotics in feed up to the first 10 weeks at the facility but after the 10 weeks, all antibiotics with residual effects were removed from feed to ensure no residual antibiotics are in the animals when they go to market. Up to the 10 weeks period, therapeutic levels of antibiotics were given to sick or injured pigs. These pigs were separated from the healthy pigs and then treated as necessary. The primary antibiotic used for treatment of these animals was penicillin, though other possible drugs for treatment include tylosin, gentamicin and tulathromycin.

After 10 weeks bacitracin was occasionally given to pigs, if needed, as this drug has no known persistent antibiotic residues in the retail meat.

Site 9: This is a Greene County finishing facility with three houses that use a flush system for waste removal into a single lagoon. It houses up to 3672 animals. Approximately 65 head of cattle grazed in a fenced field approximately 300 yards from the swine houses. Antibiotics were used as described in Site 2.

***Site 10*:** Similar to site 9, this is a Greene County Finishing facility has three houses, a single lagoon with a flush style waste removal system that houses up to 3672 pigs. This site had a fenced field to graze cattle about 150 yards from the swine houses. The farm site and pasture was separated by a row of trees and 65 head grazed this area. Antibiotic usage was as described in Site 2.

Site 11: This Green County finishing facility consists of six barns that are flushed into a single waste lagoon. It houses up to 7344 pigs. This farm grazes about 65 head of cattle in one of two fenced fields approximately 50-100 yards from the swine houses and lagoons and separated from them by a row of trees. Antibiotic usage on this farm was the same as that described in Site 2.

Site 12: This site is a sow facility located in Gates County. Sows (4800) and boars (about 30) are held at this facility year round. Once pregnancy is confirmed, there is a 112-114 day gestation period. After birth, piglets stay with their mother about 16-20 days (to about 10-12 lbs). This site has a total of eleven houses; two are farrowing houses, eight are gestation houses and one serves as an isolation barn. All eleven houses utilize a pit recharge system for waste removal into a single lagoon for primary treatment. The waste then goes to a secondary lagoon for further treatment prior to land application.

The cattle grazed on this facility are completely separate from the swine facility. The primary field was approximately 500 yards from the swine facility and across a paved road. There was also a second, smaller fenced field for grazing located approximately 400 yards from the swine facility also located on the same side of the road as the swine houses. However, during the sampling trips to this farm cattle were not grazing in this second field. There approximately 250 cattle grazed at this facility

The primary use of antibiotics in this facility was for therapeutic purposes, however, once every three months tetracycline was dosed in the feed at sub-therapeutic levels to maintain overall herd health. Therapeutic antibiotics include tetracycline, penicillin, tulathromycin and ampicillin.

Row Crop Farm Descriptions

To maintain some geographic and demographic similarities between row crop and animal agriculture sites, row crop farms were paired with one or more animal agriculture facilities. These sites were located near (generally a neighboring site) at least one of the animal agriculture facilities. The only exceptions were sites E & D. Site D was located in the same county as one of our sites but remote from it (approximately 8 miles) to ensure distance from other, non-study animal facilities. Site E was also about 8 miles from study CAFOs in Pitt County. Again the distance from study farms was required to find row crop farms with less impact from study and non-study CAFOs.

As with the animal agriculture facilities, each of the row crop farms had a non-ephemeral water body either running through it or as a border to the farm. These water bodies consisted of streams, creeks and permanent irrigation canals. Each farm varied

with regard to the crops grown, distance and direction from animal agriculture facilities and acreage.

Site A: For study purposes, this farm is associated with Sites 1 and 6 in Greene County. It is located in both Greene and Lenoir Counties. The total acreage of the farm is 950 acres, but the field sampled in this study was approximately 40 acres. The primary crops grown are cotton, corn, wheat and soybeans. Upstream samples were taken from two sites as a smaller tributary stream that flowed into the larger creek just above the downstream sample site. Therefore, the both of the potential surface water inputs (the tributary and creek) were sampled upstream of the field.

The farmer does own a small herd of cattle (about 35 head), however, these animals are grazed on a field remote from our study site.

Site B: This is a 1500 acre row crop farm located in Lenoir County near the Jones county line. For study purposes, it is associated with Site 4. Tobacco, corn, cotton and soybeans are the primary crops grown. Samples were taken upstream and downstream of the farm from permanent irrigation canals that run along the border of some fields and cut through others.

Site C: This farm is located in Greene County and is associated with Site 5 for study purposes. Corn and soybeans were the major crops grown during the sampling period. Total acreage of the farm is estimated to be 250 acres. The stream sampled ran through this farm. It is important to note that while this site was remote from our study animal agriculture facility, there was a non-study animal facility located approximately 0.25 miles from our downstream sampling site. While this animal facility was not upstream of our sampling site, it was adjacent to it and within close proximity to our

sampling site. Therefore it is possible but not likely that it impacted the bacterial quality of this sample.

Site D: This farm is located in Franklin County and is remote from any animal agriculture facilities (greater than 2 miles from any animal facilities). It consists of 150 acres on which tobacco and soybeans are primarily grown. Located on this property is a pond that serves at the head waters for a creek that flows eastward to the coast. This pond served as the upstream sample, and the creek was sampled further downstream to assess the bacterial contribution of the farm, if any, to the stream. In addition, there are three irrigation ponds adjacent to the fields. These ponds were also sampled during the study period.

***Site E*:** This farm is located in Pitt County and is associated with Sites 2, 3, 9, 10 and 11 for the purposes of this study. The total acreage of the farm is 150 acres, however, the field around which the up and downstream samples were taken was approximately 27 acres. During the time of the study, corn, soybeans and wheat were grown on the farm.

Samples were collected from a permanent irrigation canal that ran adjacent to the fields. Near the upstream sample there was a small non-study swine facility. However, this facility was upstream of the sampling site, therefore, any impact to the water would be accounted for in the upstream sample.

Site F: This row crop farm is located in Gates County. The total farm is approximately 3000 acres. However, a subsection of this farm was selected to sample. This section consisted of fields upstream of the animal agriculture facility. The stream sampled flowed through the row crop farm and then several miles downstream towards

the animal agriculture facility. The primary crops grown on this farm are cotton, corn, peanuts and soybeans.

Results

E. coli, Enterococci sp., and *Salmonella* were found in animal waste on the farms and in environmental waters. The indicator species (*E. coli* and Enterococcus) were found in all types of samples including some ground water samples; *Salmonella* were found in all types of samples except ground water samples. However, for all sample types, there were some individual samples for which *Salmonella* levels were below the detection limit.

Concentrations of Fecal Bacteria in Animal Waste and Environmental Waters by Bacterial Species

Concentrations of bacteria were estimated using Most Probable Number (MPN) methods. This method estimates the concentration based upon a maximum likelihood. As with any such method, there is uncertainty associated with the estimated value. This uncertainty is quantified using 95% confidence limits on the estimated value (not reported). For the fecal indicator bacteria, *E. coli* and Enterococci, the 95% confidence intervals were determined using the table established by IDEXX™. This table (http://www.idexx.com/water/refs/qt2k95.pdf) provides the MPN estimated value along with the associated confidence limits. For the *Salmonella* analyses, the uncertainty associated with the MPN estimate was determined using the 3- tube MPN tables provided

by FDA-BAM (2001) (http://www.cfsan.fda.gov/~ebam/bam-a2.html#excl), and adjusting for the volumes analyzed.

In addition to uncertainty in the estimated value of bacteria concentration, bacterial sampling and analysis also has inherent variability. In other words, if a sample is analyzed multiple times (e.g., triplicate) each sample may have different estimated concentrations. This variability is characterized by computing means and standard deviations. In most of the data description and statistical analyses, the geometric mean, obtained by $\log_{10}$-transforming the data (i.e. the $\log_{10}$ of the MPN value), was used to account for the variability. However, in some instances, which are indicated, the actual MPN values are used to describe the data. By using both approaches, the data are normalized (log-transformed data) which allows for more robust statistical analyses, and the extreme values can still be identified (using non-transformed data).

Animal Waste Samples

As would be expected, fecal bacteria were present in animal waste samples. Concentrations of the fecal indicator bacteria were present in higher concentrations than those of the frank pathogen *Salmonella*. The fecal indicator bacteria were present in all samples. *Salmonella* were detected in most, but not all, waste samples.

E. coli concentrations were generally high in the animal waste samples The geometric mean concentrations were as high as 7.7 $\log_{10}$ cfu/100ml in waste. The overall geometric means in the lagoons (pooled), barns and cow manure samples were 4.7, 6.5 and 7.0 $\log_{10}$, respectively (table 4.2).

Table 4.2: Log_{10} (MPN) *E. coli* Concentrations per 100ml in Animal Waste Samples

Season	Sample	n	Mean	Std Dev	Min	Max
Cool	1º Lagoon	14	5.3	0.6	4.0	6.1
	2º Lagoon	2	4.9	0.6	4.5	5.3
	3º Lagoon	1	5.3	--	--	--
	All Lagoons	17	5.2	0.6	4.0	6.1
	Barn	13	6.5	0.9	4.8	8.2
	Cow Manure	8	7.1	0.7	6.2	8.2
Moderate	1º Lagoon	14	4.7	0.4	4.1	5.6
	2º Lagoon	2	4.3	0.4	4.0	4.6
	3º Lagoon	1	4.6	--	--	--
	All Lagoons	17	4.7	0.4	4.0	5.6
	Barn	13	6.5	0.95	5.0	7.8
	Cow Manure	9	7.7	0.7	6.8	8.9
Warm	1º Lagoon	20	4.6	0.5	4.0	5.2
	2º Lagoon	3	3.6	0.4	3.3	4.0
	3º Lagoon	1	4.0	--	--	--
	All Lagoons	24	4.4	0.5	3.3	5.2
	Barn	18	6.4	0.9	5.0	8.0
	Cow Manure	14	6.5	0.9	4.9	8.2
Overall	1º Lagoon	48	4.8	0.6	4.0	6.1
	2º Lagoon	7	4.2	0.7	3.3	5.3
	3º Lagoon	3	4.6	0.6	4.0	5.3
	All Lagoons	58	4.7	0.6	3.3	6.1
	Barn	44	6.5	0.9	4.8	8.2
	Cow Manure	31	7.0	0.9	4.9	8.9

The highest *E. coli* concentrations were found in the cow manure samples with swine barn flush samples being the next highest. There was a reduction in the concentration of *E. coli* found in swine lagoons (all types pooled) compared to that found in the barn flush samples. When the geometric means of these samples were compared using an unpaired t-test analysis, the difference was found to be statistically significant with a p-value of <0.0001. This indicates that the lagoon system does reduce bacteria to some extent. And in the farms analyzed this reduction is approximately 2 $\log_{10}$.

Enterococcus concentrations were slightly lower than those for *E. coli*. However, as with the *E. coli*, the highest concentrations generally were found in the barn flush and cow manure samples, with overall geometric means of 6.6 $\log_{10}$ cfu/100ml for each Enterococcus concentrations in lagoon liquid were lower than in barn flush (pooled lagoon overall mean = 4.9 $\log_{10}$ cfu/100ml), indicating an approximate 1.7 $\log_{10}$ reduction (Table 4.3). As with the *E. coli*, the difference in Enterococcus concentrations in the barn flush samples compared with those in the lagoons is statistically significant (unpaired t test – p-value <0.0001).

Table 4.3: Log_{10} (MPN) Enterococcus Concentration per 100ml in Animal Waste Samples

Season	Sample	n	Mean	Std Dev	Min	Max
Cool	1º Lagoon	14	5.5	0.6	4.6	6.8
	2º Lagoon	2	4.1	0.3	3.9	4.3
	3º Lagoon	1	4.3	--	--	--
	All Lagoons	17	5.3	0.8	3.9	6.8
	Barn	13	6.8	0.9	5	8.1
	Cow Manure	8	6.4	0.9	5	7.96
Moderate	1º Lagoon	14	4.9	0.4	4.0	5.8
	2º Lagoon	2	3.6	1.1	2.9	4.4
	3º Lagoon	1	4	--	--	--
	All Lagoons	17	4.7	0.7	2.9	5.8
	Barn	13	6.5	1.0	5	8.2
	Cow Manure	9	7.3	0.6	6.4	8.3
Warm	1º Lagoon	20	4.9	0.7	4	6.4
	2º Lagoon	3	4.1	0.8	3.1	4.7
	3º Lagoon	1	4.8	--	--	--
	All Lagoons	24	4.8	0.7	3.1	6.4
	Barn	18	6.5	1.0	4.7	8.4
	Cow Manure	14	6.3	0.7	5.1	7.6
Overall	1º Lagoon	48	5.1	0.6	4	6.8
	2º Lagoon	7	3.9	0.7	2.9	4.7
	3º Lagoon	3	4.4	0.4	4	4.8
	All Lagoons	58	4.9	0.8	2.9	6.8
	Barn	44	6.6	0.98	4.7	8.4
	Cow Manure	31	6.6	0.8	5	8.3

Salmonella concentrations in the animal waste samples were much lower than that of the indicator bacteria. *Salmonella* was detectable in most swine waste samples: 85% of primary lagoon samples, 71% of secondary lagoons, and 70% of barn flush samples. For these samples, the lower detection limit was relatively high, at 30cfu/100ml for barn flush samples and 3cfu/100ml for lagoon samples. Therefore, it is possible that there were in fact *Salmonella* in these samples but below the detectable level of the assay method.

Salmonella was not as prevalent in cattle manure samples; only 35% had detectable levels of *Salmonella* (lower detection limit is 0.03cfu/g). However, in samples where *Salmonella* was detected, concentrations were as high as $3.5\log_{10}$ cfu/g.

Overall, *Salmonella* concentrations in swine waste samples were greater than 2.4 $\log_{10}$ in untreated waste, and 1.5 $\log_{10}$ in wastes treated in a lagoon. *Salmonella* concentrations were further reduced by secondary lagoon treatment, resulting in an overall geometric mean concentration of 1.1 $\log_{10}$ (Table 4.4). The differences in the *Salmonella* concentration of barn flush and the primary treatment lagoon liquids were considered statistically significant with a p value of <0.0001. For this comparison however, the non-parametric Mann-Whitney test was used because even with $\log_{10}$-transformation the assumptions of normality for the distribution of the *Salmonella* concentrations was not met. Further *Salmonella* reductions by secondary treatment were not subjected to statistical analyses, due to the fact that there were too few samples for a robust comparison.

Table 4.4: Log_{10} (MPN) *Salmonella* Concentration per 100ml in Animal Waste Samples

Season	Sample	N	Mean	Std Dev	Min	Max
Cool	1º Lagoon	14	1.9	1.2	0.5	4.0
	2º Lagoon	2	1.6	1.5	0.5	2.7
	3º Lagoon	1	0.5	--	--	--
	All Lagoons	17	1.7	1.2	05	4.0
	Barn	13	2.1	0.7	1.5	3.9
	Cow Manure	8	-1.5	0.2	-1.5	-1.0
Moderate	1º Lagoon	14	1.4	0.9	0.5	3.7
	2º Lagoon	2	0.7	0.2	0.6	0.9
	3º Lagoon	1	0.6	--	--	--
	All Lagoons	17	1.3	0.6	0.5	3.7
	Barn	13	2.4	1.3	1.5	4.7
	Cow Manure	9	-1.2	0.7	-1.5	0.3
Warm	1º Lagoon	20	1.4	0.6	0.5	4.0
	2º Lagoon	3	0.9	0.4	0.5	1.4
	3º Lagoon	1	0.5	--	--	--
	All Lagoons	24	1.3	0.6	0.5	2.6
	Barn	18	2.4	1.1	1.5	5.0
	Cow Manure	14	-0.2	2.1	-1.5	3.5
Overall	1º Lagoon	48	1.5	0.9	0.5	4.0
	2º Lagoon	7	1.1	0.8	0.5	2.7
	3º Lagoon	3	0.5	0.05	0.5	0.6
	All Lagoons	58	1.4	0.9	0.5	4.0
	Barn	44	2.3	1.2	1.5	5.0
	Cow Manure	31	-0.8	1.5	-1.5	3.5

Comparisons of bacteria concentrations by individual farm

Pooling the data by season and comparing log_{10} MPN bacteria concentrations by farm, there were some variations in the bacterial concentrations found in animal waste samples. Using the Kruskal-Wallis Rank test, the variation between farms was assessed. It was found that among the fecal indicator bacteria there were significant differences in the log_{10} concentrations of some bacteria found in some of the waste samples (table 4.5 a

& b). *E. coli* concentrations varied between farms in barn flush samples (p= 0.0016) but there were no significant differences in *E. coli* concentrations between farms in any other samples. Enterococcus concentrations varied by farm in barn flush samples as well as the lagoons when pooled; however, analyses by type of lagoon revealed no significant differences.

Analyzing the *Salmonella* concentrations revealed that there were no significant differences in concentration by farm in any of the waste samples except secondary lagoons (figure 4.5 c). It is important to note however, that only two of the eleven farms had secondary lagoons and therefore, this difference may in fact be an artifact of low sample size.

Table 4.5: Kruskal-Wallis Rank Test Probability Values Comparing Bacterial Concentrations in Animal Waste Samples by Farm
(a. *E. coli* b. *Enterococci*, and c. *Salmonella*)

a. *E. coli*

Samples compared	Probability
All Lagoons	0.1183
Primary Lagoon	0.0926
Secondary Lagoon	0.0771
Barn Flush	0.0016
Cow Manure	0.5073

b. *Enterococci*

Samples compared	Probability
All Lagoons	0.0014
Primary Lagoon	0.0754
Secondary Lagoon	0.2888
Barn Flush	0.0277
Cow Manure	0.6875

c. *Salmonella*

Samples compared	Probability
All Lagoons	0.1409
Primary Lagoon	0.3404
Secondary Lagoon	0.0339
Barn Flush	0.7414
Cow Manure	0.1957

Summary

Overall, concentrations of fecal bacteria on the farms were high (geometric mean values >6log10 cfu/100ml for indicator bacteria and >2log cfu/100ml of *Salmonella*). While there is a significant reduction of the bacteria from treatment in the waste lagoons, the lagoons still have concentrations high enough to have the potential of cause exposures if these bacteria are released into the environment and people come in contact with lagoon liquid directly or indirectly. Concentrations of *Salmonella* were as high as 4.0 $\log_{10}$ cfu/100ml. *Salmonella* can cause infection and illness in low doses (Koch, J., et al., 2005). While it is not likely that concentrations would remain this high when dilution factors in the stream waters are considered, if there were a catastrophic event such as a hurricane, or massive flooding, it is possible that these lagoons and other animal waste could enter environmental water bodies at or near these high concentrations in lagoon liquid.

There are some differences in fecal indicator species in swine wastes by farm. Most of these differences in farm-to-farm concentrations were in the fresh feces (barn flush) samples and the overall concentrations are less variable in the treatment lagoons. These variations in the barn flush samples could be due to inter-animal variability or the variable extent of swine feces dilution by the barn flush procedures.

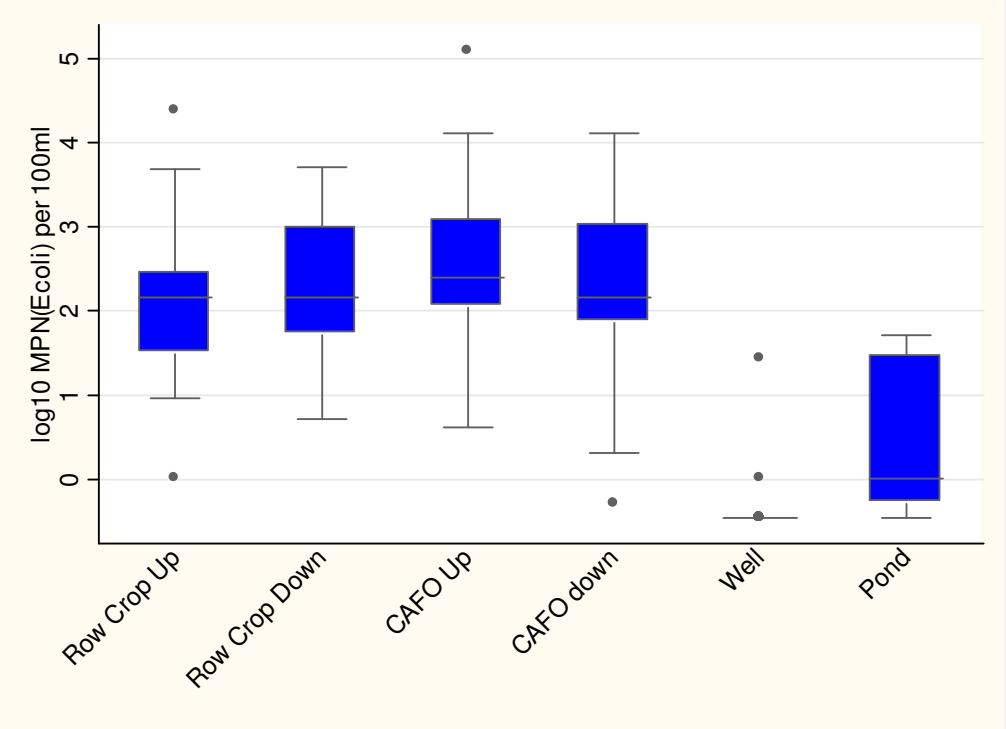
Water Samples

Overall, bacterial concentrations in environmental water were much lower than those found in animal waste samples on the farms. The highest geometric mean

concentration of any of the three bacterial genus/species in stream water was 2.4 $\log_{10}$ cfu/100ml, compared with the highest waste concentration of 7.0$\log_{10}$cfu/100ml. Concentrations in ground water were even lower. The majority of ground waters 79% did not have any bacteria at detectable levels (lower detection limit of 0.03cfu/100ml); and there were no ground water samples in which *Salmonella* was detected.

E. coli: In all seasons, detectable *E. coli* concentrations were found in all samples, except for ground water samples in which only two samples (5%) had detectable levels of them. The concentrations of the *E. coli* in the irrigation ponds associated with one of the row crop farms were lower than those found in the stream water samples (figure 4. 1). The geometric mean *E. coli* concentration of stream water samples (pooled) is 2.3$\log_{10}$ cfu/100ml, while the geometric mean of the pond samples is 0.4$\log_{10}$cfu/100ml. Comparing the two groups using the Mann-Whitney test, it was determined that the two medians are statistically different. However, it is important to note that the irrigation ponds were associated with only one row crop farm and each of the three ponds were only sampled three times.

Figure 4.1: Log_{10} (MPN) *E. coli* Concentrations per 100ml in Environmental Water Samples

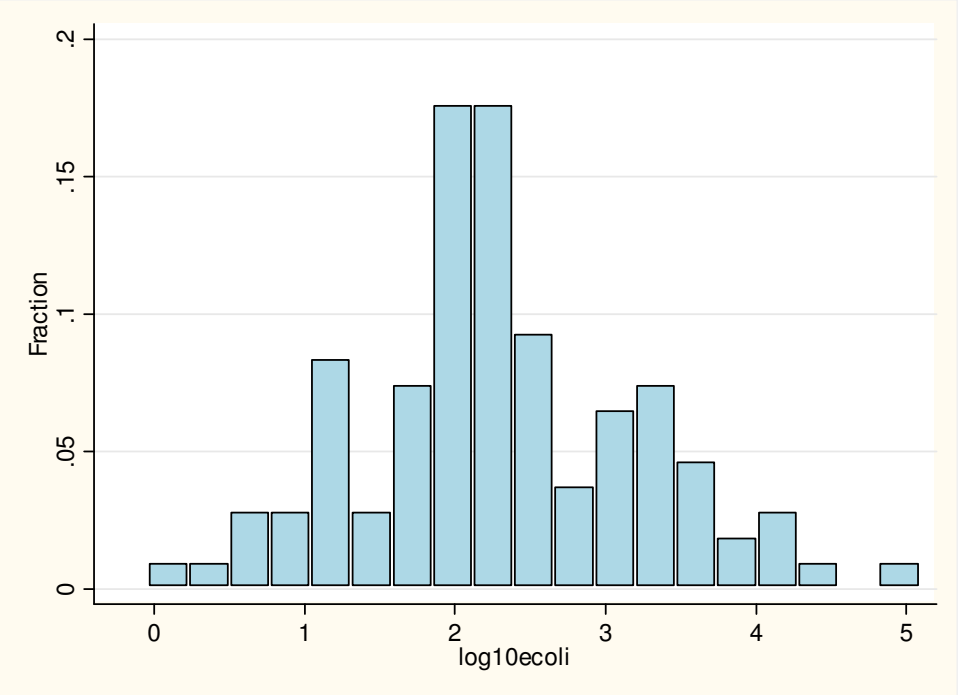


While the overall geometric mean in the stream water samples (pooled) was 2.3 log_{10}, concentrations were found as high as 5.1 log_{10}cfu/100ml in the warm season and as low as -0.3 log_{10} in the cool season (table 4.6). Overall, the log_{10}-transformed data were normally distributed with a slight skew to the right (skew =0.145) and were slightly more peaked than normal (kurtosis = 3.43 compared with the normal kurtosis of 3.0) (figure 4.2.

Table 4.6: Log_{10} (MPN) *E. coli* concentration per 100ml in Water Samples

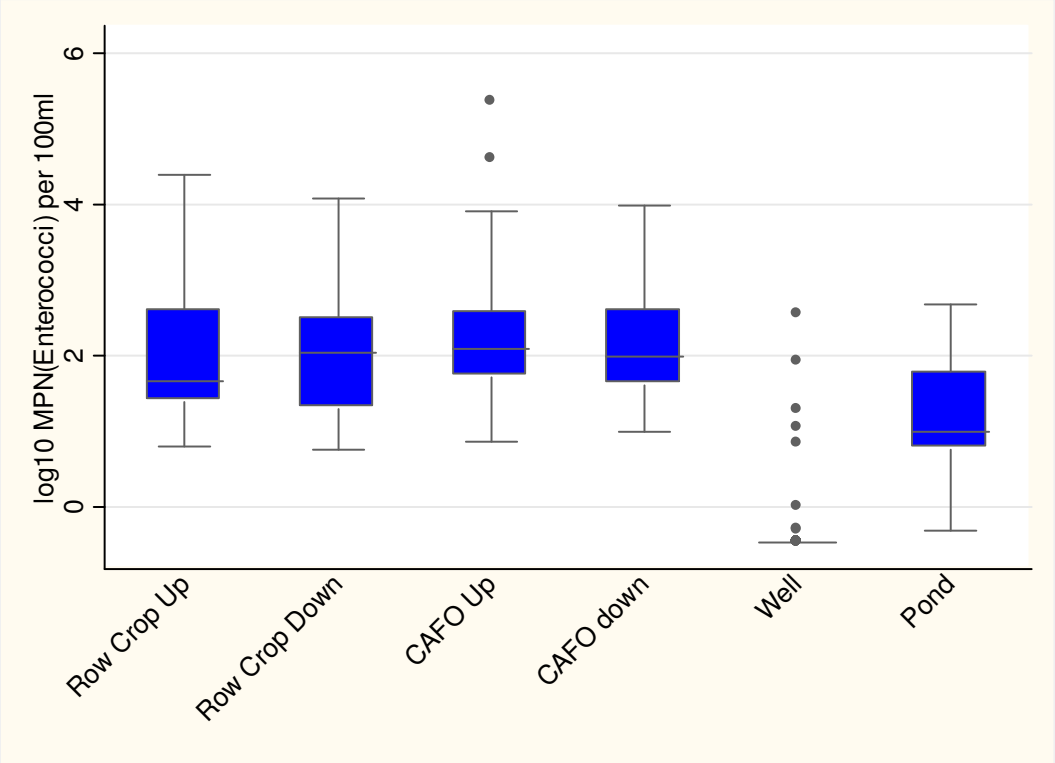
Season	Sample	n	Mean	Std Dev	Min	Max
Cool	Stream	32	2.0	0.9	-0.3	3.8
	Pond	3	-0.4	0.1	-0.5	-0.3
	Well	12	-0.5	0	-0.5	-0.5
Moderate	Stream	34	2.3	0.7	0.96	4.1
	Pond	3	.005	0.3	-0.3	0.3
	Well	12	-0.26	0.5	-0.5	1.4
Warm	Stream	43	2.5	1.1	0.3	5.1
	Pond	3	1.6	0.1	1.5	1.7
	Well	18	-0.5	0	-0.5	-0.5
Overall	Stream	109	2.3	0.9	-0.3	5.1
	Pond	9	0.4	0.9	-0.5	1.7
	Well	42	-0.4	0.3	-0.5	1.4

Figure 4.2: Overall Frequency Distribution of Log_{10} (MPN) *E. coli* Concentration per 100ml in Stream Samples



Enterococcus: As with *E. coli*, detectable levels of *Enterococcus* sp were found in all surface water samples. *Enterococcus* sp. were also found in several well water samples in low concentrations, especially in the warm season. Furthermore, the *Enterococcus* sp. concentrations in the pond samples were higher than the concentrations of *E. coli* (figure 4.3); with an overall geometric mean of 1.2 $\log_{10}$ cfu/100ml compared with 0.4$\log_{10}$ cfu/100ml in the *E. coli*. This difference was considered statistically significant using a paired t test (p value =<0.0001).

Figure 4.3: Log_{10} (MPN) *Enterococci* Concentrations per 100ml in Environmental Water Samples



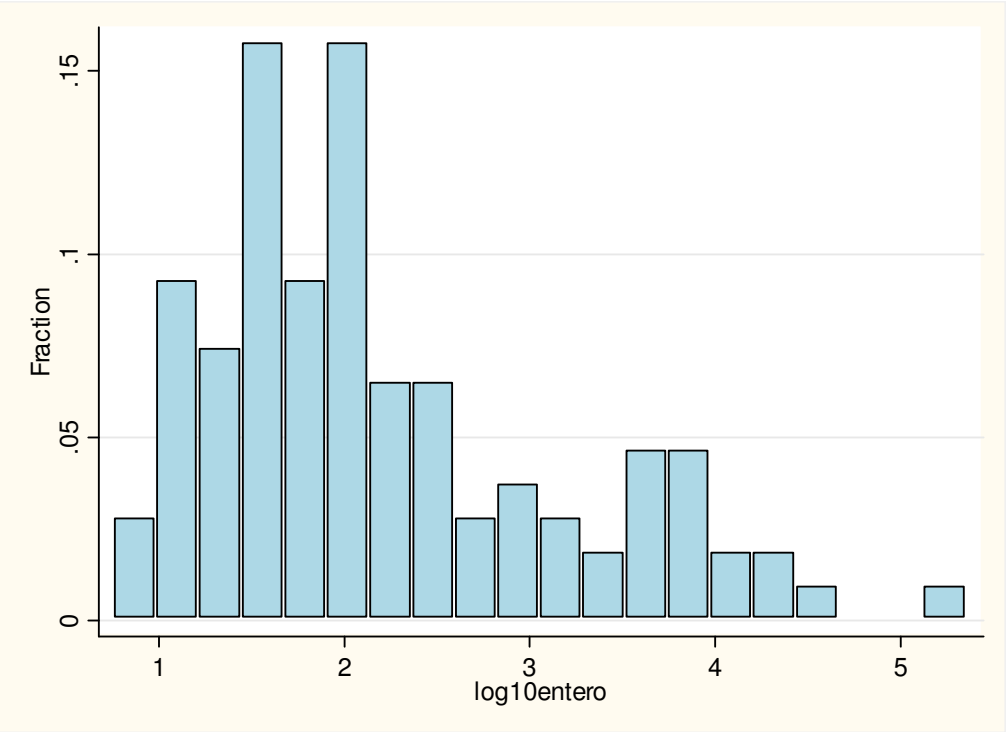
Enterococcus sp. concentrations in surface water were as high as 5.4 $\log_{10}$ cfu/100ml, and as low as -0.5 $\log_{10}$cfu/100ml. Pond samples were as high as 2.7 $\log_{10}$

cfu/100ml, and concentrations in well water were as high as 2.6 $\log_{10}$ cfu/100ml (Table 4.7). The log-transformed data were more skewed than that of the *E. coli* (skew=0.89) and slightly less peaked (kurtosis = 3.20) (figure 4.4). However, the geometric means of the two types of bacteria were about the same, 2.3 $\log_{10}$cfu/100ml (*E. coli*) and 2.2$\log_{10}$cfu/100ml (Enterococci).

Table 4.5: Log_{10} *Enterococcus* concentration per 100ml in Water Samples

Season	Sample	n	Mean	Std Dev	Min	Max
Cool	Stream	32	1.9	0.95	0.8	3.99
	Pond	3	0.6	1.0	-0.3	1.7
	Well	12	-0.5	0	-0.5	-0.5
Moderate	Stream	34	1.9	0.7	1	3.96
	Pond	3	0.8	0.1	0.8	1
	Well	12	-0.3	0.4	-0.5	0.8
Warm	Stream	43	2.7	0.99	1	5.4
	Pond	3	2.2	0.4	1.8	2.7
	Well	18	0.06	0.96	-0.5	2.6
Overall	Stream	109	2.2	0.98	0.8	5.4
	Pond	9	1.2	0.9	-0.3	2.7
	Well	42	-0.20	0.69	-0.5	2.6

Figure 4.4: Overall Frequency Distribution of Log10 (MPN) Enterococcus Concentration per 100ml in Stream Samples

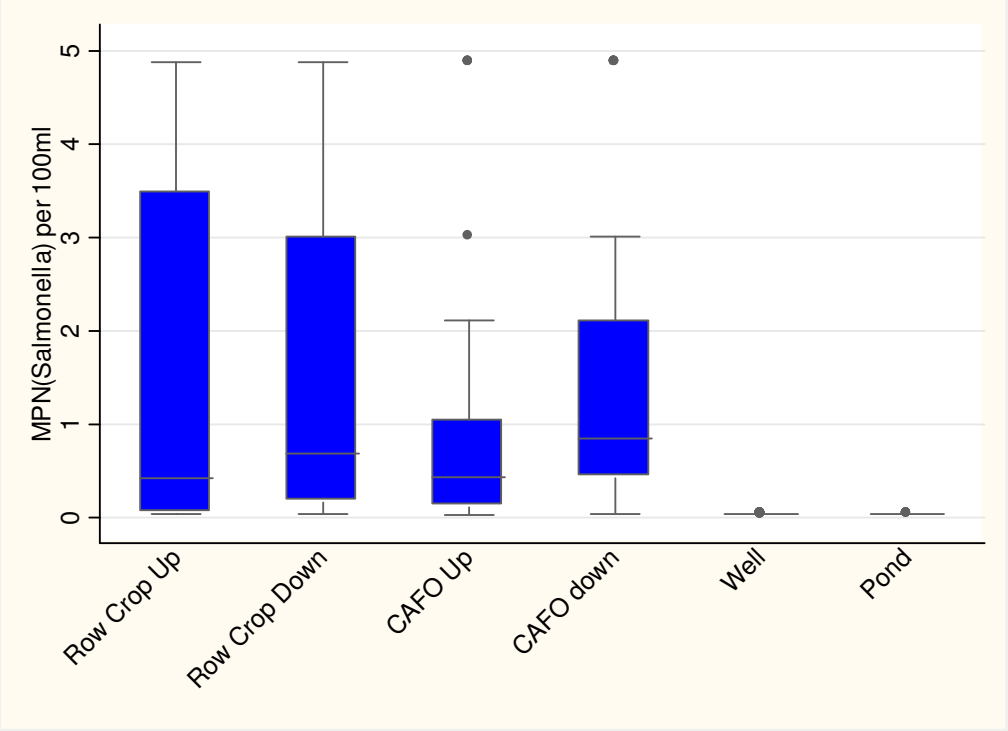


Salmonella: Unlike the indicator bacteria, *Salmonella*, a human pathogen, was not found in all samples (Table 4.8). For the *Salmonella* analyses, there was a lower detection limit of -1.5 $\log_{10}$ per 100 ml, (or 0.03 cfu/100ml), and an upper detection limit of 0.7 $\log_{10}$ per 100 ml (4.87 cfu/100ml). No *Salmonella* were found in 15.6% of stream samples (occurring in all seasons and samples), in all but one of the pond samples and in all of the ground water samples. For the purposes of analyses, the minimum detection limit is used, but it must be noted that this is in fact a less than value. Therefore, it is possible that mean values are lower than recorded. In some instances (15.6 % or 17 samples), the maximum detection value was reached. As with the minimum detection

values, this value upper detection limit was used in analysis, and therefore, it is possible that estimated mean concentrations are in fact higher than reported.

In the majority of environmental surface water samples, S*almonella* were found. Approximately 84% of stream water samples had detectable levels of S*almonella*, of which 45% had concentrations less than 1 cfu/100ml and 39% had concentrations greater than 1 cfu/100ml. Only one pond sample had detectable levels of *Salmonella* and it was a very low concentration of 0.04cfu/100ml. There were no *Salmonella* found in any ground water samples (figure 4.5).

Figure 4.5: Salmonella (MPN) Concentrations per 100ml in Environmental Water Samples*



*NOTE: This graph is not $\log_{10}$-transformed; data are actual MPN estimates of concentration

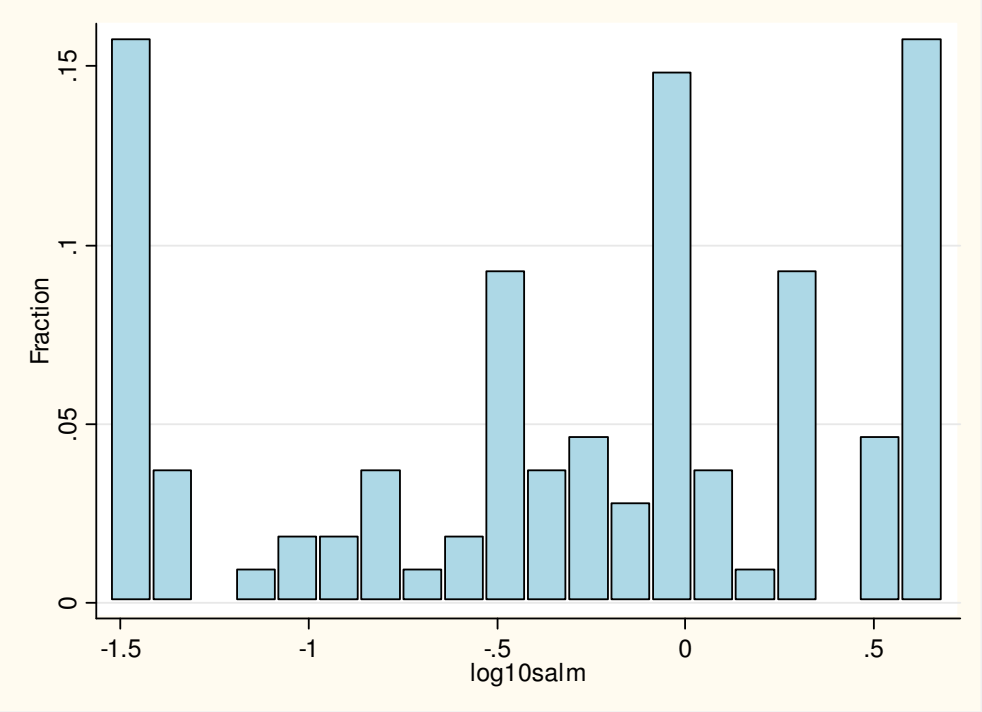
The geometric mean concentration of *Salmonella* in all stream samples was -0.3$\log_{10}$cfu/100mls. However, this value may be slightly misleading as there were samples for which the concentrations were at the upper (0.7$\log_{10}$cfu/100ml) or lower (-1.5$\log_{10}$cfu /100ml) detection limits (table 4.8).

Table 4.8: Log10 Salmonella concentration per 100ml in Water Samples

Season	Sample	N	Mean	Std Dev	Min	Max
Cool	Stream	32	-0.4	0.8	-1.5	0.7
	Pond	3	-1.5	0	-1.5	-1.5
	Well	12	-1.5	0	-1.5	-1.5
Moderate	Stream	34	-0.3	0.7	-1.5	0.7
	Pond	3	-1.5	0	-1.5	-1.5
	Well	12	-1.5	0.04	-1.5	-1.4
Warm	Stream	43	-0.2	0.7	-1.5	0.7
	Pond	3	-1.5	0.04	-1.5	-1.4
	Well	18	-1.5	0.03	-1.5	-1.4
Overall	Stream	109	-0.3	0.7	-1.5	0.7
	Pond	9	-1.5	0.03	-1.5	-1.4
	Well	42	-1.5	0.03	-1.5	-1.4

The *Salmonella* concentrations, even after $\log_{10}$-transforming the data did not approximate a normal distribution. It has a negative skew of -0.36 and is not peaked with a kurtosis of only 1.94 (figure 4.6).

Figure 4.6: Overall Frequency Distribution of Log_{10} (MPN) *Salmonella* Concentration per 100ml in Stream Samples



Data analyses

Comparison of Stream Water Samples

When analyzing the stream samples for bacteria concentration at the four different sampling site (i.e. up or downstream of each of the two types study farms), the overall geometric means at each sample site are similar (between 2.2 ad 2.5 log_{10}/100 ml), although the skew and kurtosis vary among the different groups (Table 4.9 and Figures 4.7, 4.8, 4.9).

Table 4.9a, b, c: Log_{10} (MPN) *E. coli* (a), *Enterococci* (b) and *Salmonella* (c) concentrations per 100ml by sampling site

a. *E. coli*

Sampling Site	n*	Mean† (StD)	Min	Max	Skew	Kurtosis
Row crop Upstream	24	2.12 (0.95)	0.004	4.38	0.233	3.44
Row Crop Downstream	21	2.20 (0.87)	0.716	3.7	0.078	2.11
CAFO Upstream	26	2.5 (1.03)	0.619	5.10	0.502	3.11
CAFO Downstream	37	2.26 (0.91)	-0.30	4.11	-0.404	3.60

b. Enterococci

Sampling Site	n*	Mean† (StD)	Min	Max	Skew	Kurtosis
Row crop Upstream	24	2.06 (1.03)	0.80	4.38	0.97	2.90
Row Crop Downstream	21	2.09 (0.98)	0.76	4.08	0.59	2.31
CAFO Upstream	26	2.37 (1.07)	0.87	5.36	1.22	3.98
CAFO Downstream	37	2.20 (0.90)	1	3.99	0.64	2.37

c. *Salmonella*

Sampling Site	n*	Mean† (StD)	Min	Max	Skew	Kurtosis
Row crop Upstream	24	-0.40 (0.88)	-1.48	0.69	-0.06	1.46
Row Crop Downstream	21	-0.27 (0.76)	-1.48	0.69	-0.25	1.71
CAFO Upstream	26	-0.42 (0.72)	-1.48	0.69	-0.13	1.96
CAFO Downstream	37	-0.14 (0.62)	-1.48	0.69	-0.82	3.1

*In some instances a downstream sample for one farm was an upstream sample for another. In these instances the value is reported in the downstream sample, except in paired analyses. Also for one row crop farm a second tributary entered the stream prior to the downstream sample, therefore there are two upstream samples taken.

† geometric mean value

Figure 4.7: Frequency Distributions of Log_{10} (MPN) *E. coli* Concentration per 100ml by Stream Sample Type

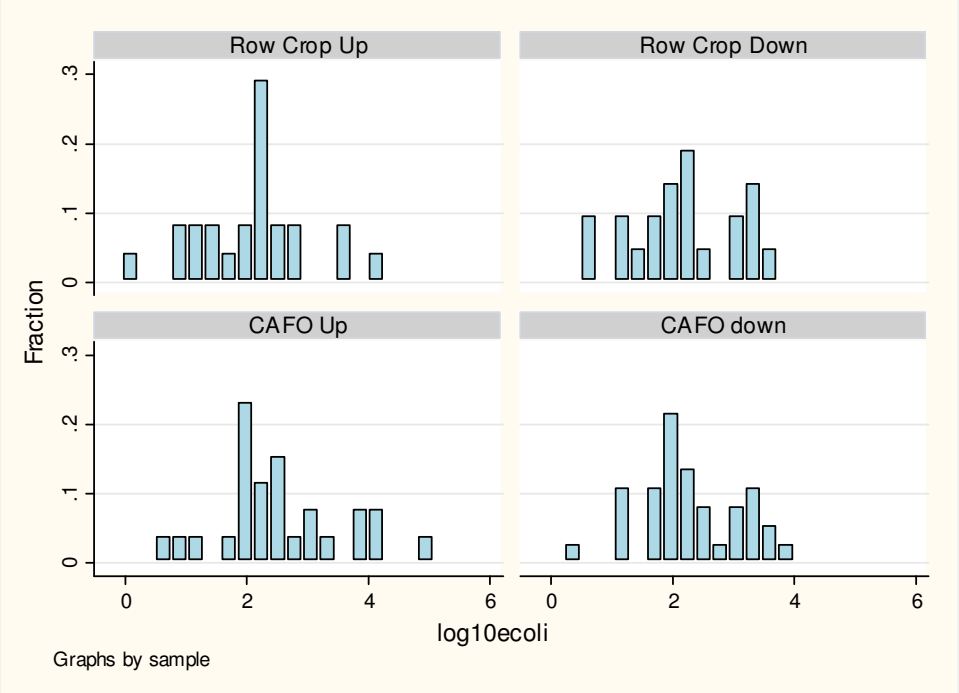


Figure 4.8: Frequency Distributions of Log_{10} (MPN) *Enterococci* Concentration per 100ml by Stream Sample Type

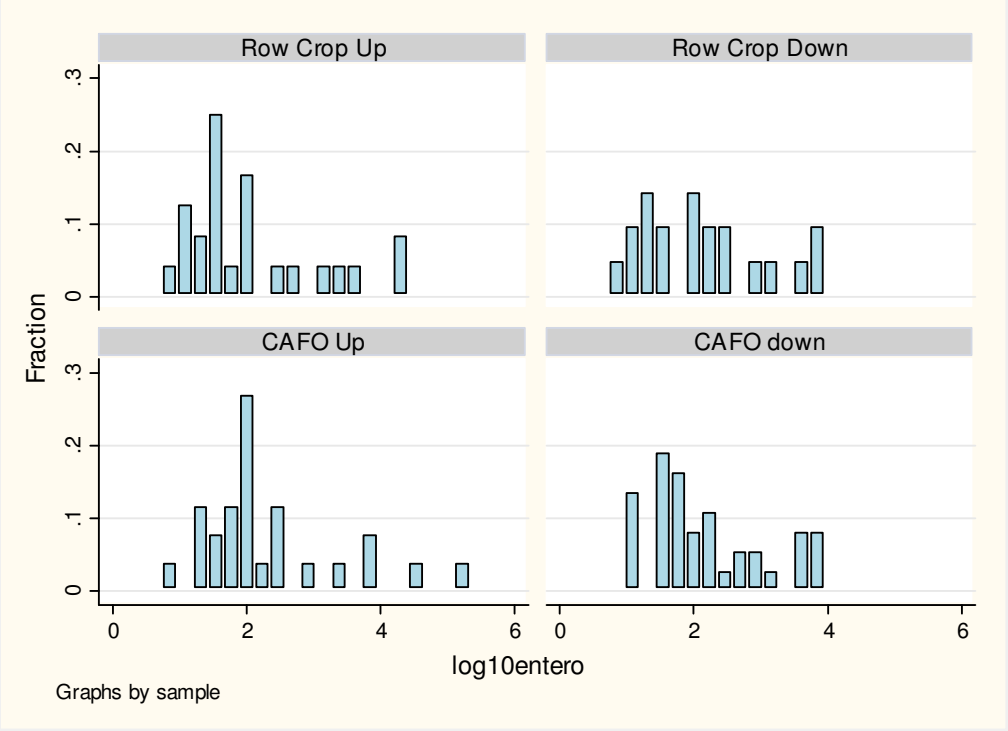
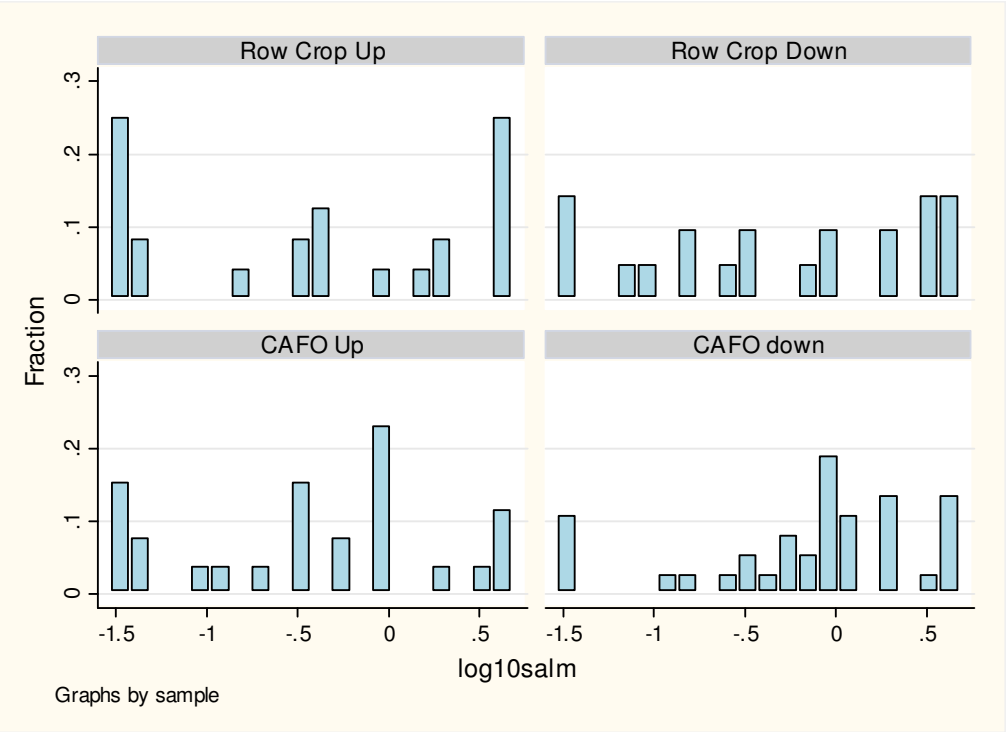


Figure 4.9: Frequency Distributions of Log_{10} (MPN) *Salmonella* Concentration per 100ml by Stream Sample Type



Statistical Comparisons

The impact of farms on bacterial concentrations in water as well as the impact of the type of farm (animal agriculture compared with non-animal agriculture) was explored by several methods of statistical analyses. These methods included comparisons based upon geometric mean concentrations the arithmetic mean concentrations, and using the 90^{th} percentile or the maximum concentration in the data set (LeChevallier, M.W. and Norton, W.D., 1995). Additionally, non-parametric tests were employed using the median values to analyze non-normal distributions or data that do not meet required assumptions.

The first set of comparisons was designed to understand the impacts of individual on concentrations of bacteria in water. The initial statistical test employed was a paired t test. This test was used to assess the differences in bacteria concentrations of water upstream of the farms compared with that downstream of the farm, by farm type (i.e. row crop upstream samples were compared with row crop downstream samples, and upstream CAFO samples were compared with CAFO downstream samples). For each sampling date, the upstream and downstream samples at each farm type were paired, the differences in concentration were assessed and the geometric means of the differences in the $\log_{10}$ concentrations by farms were determined.

For both farm types, there were no statistically significant differences found in concentration of fecal indicator bacteria between up and down steam samples. There were no differences in *Salmonella* concentrations in upstream and downstream waters of row crop farms, however, there was a statistically significant increase in the concentrations of *Salmonella* in water downstream of the CAFO facilities compared with waters upstream (table 4. 10). This finding may not be reliable however, as the normality assumption for the distribution of *Salmonella* in the CAFO downstream samples was not met. Using a less robust but more appropriate non-parametric method of analysis (the Wilcoxon Matched Pairs test) to compare the differences in the median $\log_{10}$-transformed concentrations of *Salmonella*, the p-value was 0.0516. This was not quite but nearly significant for α=0.05.

Table 4.10: P values and differences and mean difference in the log_{10} concentrations of bacteria found up and downstream of row crop farms and CAFOs using paired t test analyses

organism	Farm type	n	p-value	Mean difference*
E. coli	Row Crop	21	0.991	-.0002
E. coli	CAFO	37	0.7953	0.035
Enterococcus	Row Crop	21	0.8337	0.033
Enterococcus	CAFO	37	0.6742	-0.06
Salmonella	Row Crop	21	0.5435	-0.1195
Salmonella	CAFO	37	0.0390	-0.257

* This is the geometric mean of the differences in the log_{10} values of the bacteria by pair

The second comparison of interest was the difference in bacterial concentrations downstream of row crop farms compared with concentrations downstream of swine CAFOs. For these comparisons, it was necessary to use non-paired analyses. The first test used to compare was the unpaired t test (table 4.11). There were no significant differences in the concentrations of any of the three types of bacteria found in downstream samples when comparing swine CAFOs to row crop farms. However, as with the comparisons within farm types, comparing *Salmonella* concentrations downstream of swine CAFOs to *salmonella* downstream of row crop farms required a nonparametric test, as the normality assumption for these data was not met. Using the Mann-Whitney test to compare the median concentrations of *Salmonella* found in downstream water samples of swine CAFOS and row crop farms, there still was no statistically significant difference (p-value = 0.5546), observed.

Table 4.11:P –values from Unpaired t-test Analyses Comparing Downstream samples of CAFOs and Row Crop Farms

Organism	p-value
E. coli	0.8149
Enterococci	0.6687
Salmonella	0.4579*

*normality assumptions not met sample was reanalyzed using the nonparametric Mann-Whiney test resulting p-value = 0.5546

In addition to the unpaired t-test and the non parametric equivalent, the Mann-Whitney test, linear regression was used to analyze these data. In this case, a binary variable was created and coded as 1 for the "exposure" group of concern (CAFOs) and coded 0 if the sample was associated with the "control" group (row crop farms). A basic linear regression for a Gaussian outcome (bacterial concentration) was used as follows:

Probability(bacterial conc) = α + β(farm association) **eqn 4- 4**

The model was employed using both arithmetic and $\log_{10}$transformed values. In these analyses, it was found that there were no statistically significant differences in bacterial concentrations downstream of the two different farm types (CAFO compared with row crop) for any of the three bacteria (table 4.7). Furthermore, the very low (<0.01) R^2 values associated with these analyses indicate very little if any correlation with sampling site and bacterial concentration.

Table 4.12:P-values and Correlation Coefficients Based upon Linear Regression for Gaussian Outcomes Using MPN Values and Log_{10}-Transformed Data of Bacterial Concentrations as Outcome Variables

Organism	Outcome (MPN/Log_{10}(MPN)	P – value	R^2
E. coli	MPN	0.694	0.0028
E. coli	Log_{10}(MPN)	0.815	0.0010
Enterococci	MPN	0.965	0.0000
Enterococci	Log_{10}(MPN)	0.669	0.0033
Salmonella	MPN	0.890	0.0003
Salmonella	Log_{10}(MPN)	0.458	0.0099

A final analysis that was conducted to compare concentrations in the stream samples was to examine the number of samples collected by sample type that were at or above the 90th percentile. This approach is similar to that used by LeChevallier and Norton (1995). The advantage of this analysis is that it provides information with regard

to the highest concentrations seen rather than the mean or median values. Especially in dealing with pathogens, which may have a low infectious dose, it is important to identify where these higher concentrations occur and determine if they are associated with one farm type or if high values occur in both locations.

For each of the three types of bacteria, the 90th percentile for the concentration was determined. For *E. coli*, 90% of the water samples taken had an estimated concentration of 3450cfu/100ml or lower. For Enterococcus sp., 90% of the water samples had an estimated concentration less than or equal to 6200cfu/100ml. The 90th percentile for *Salmonella* was the upper detection limit. Using the 90th percentile as a bench mark, the cumulative percent of samples per sampling site (row crop upstream (RCU), row crop downstream (RCD), CAFO upstream (SWU) and CAFO downstream (SWD)) were plotted with the concentration at the percent. The number of samples at each sampling site at or above the overall 90th percentile were then compared to see if there were any differences in the occurrence of these maximum values by sample type (figures 4. 10 , 4. 11 , 4.12). The y axis of these figures is on the logarithmic scale, however, the value plotted are the arithmetic MPN estimated concentrations.

Figure 4.10: Concentration of *E. coli* in Water Samples by Sample Type as a Function of Cumulative Percent Compared with the Overall 90th Percentile

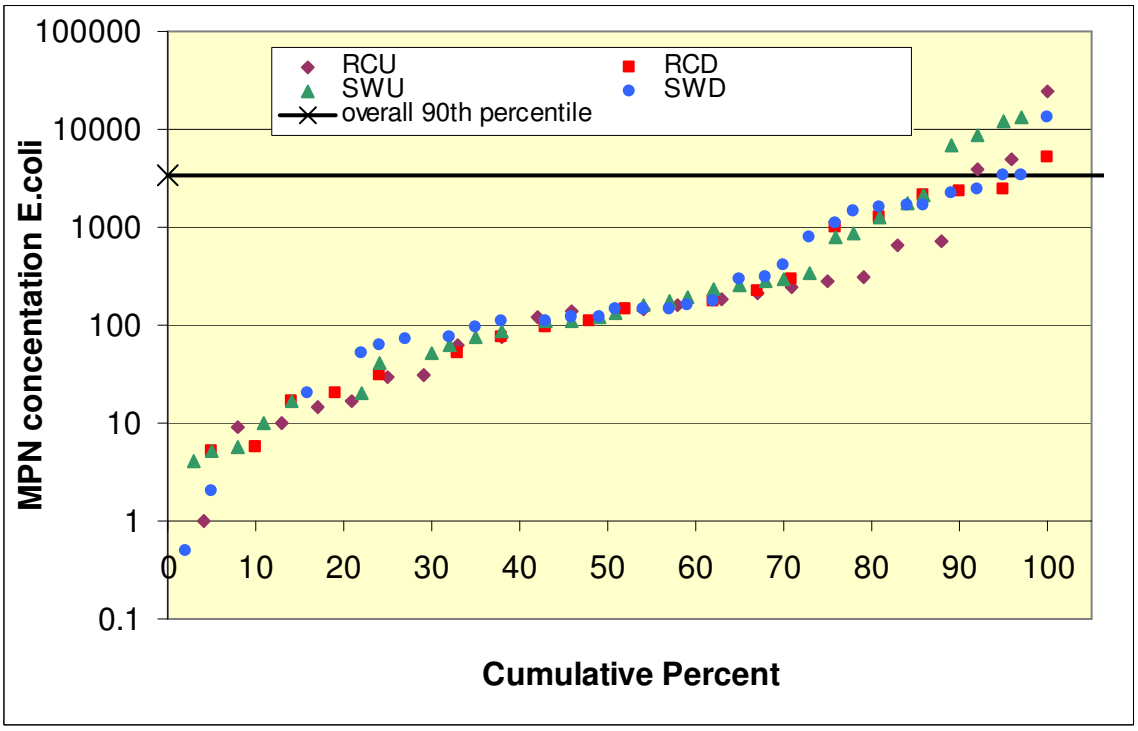


Figure 4.11: Concentration of *Enterococci* in Water Samples by Sample Type as a Function of Cumulative Percent Compared with the Overall 90th Percentile

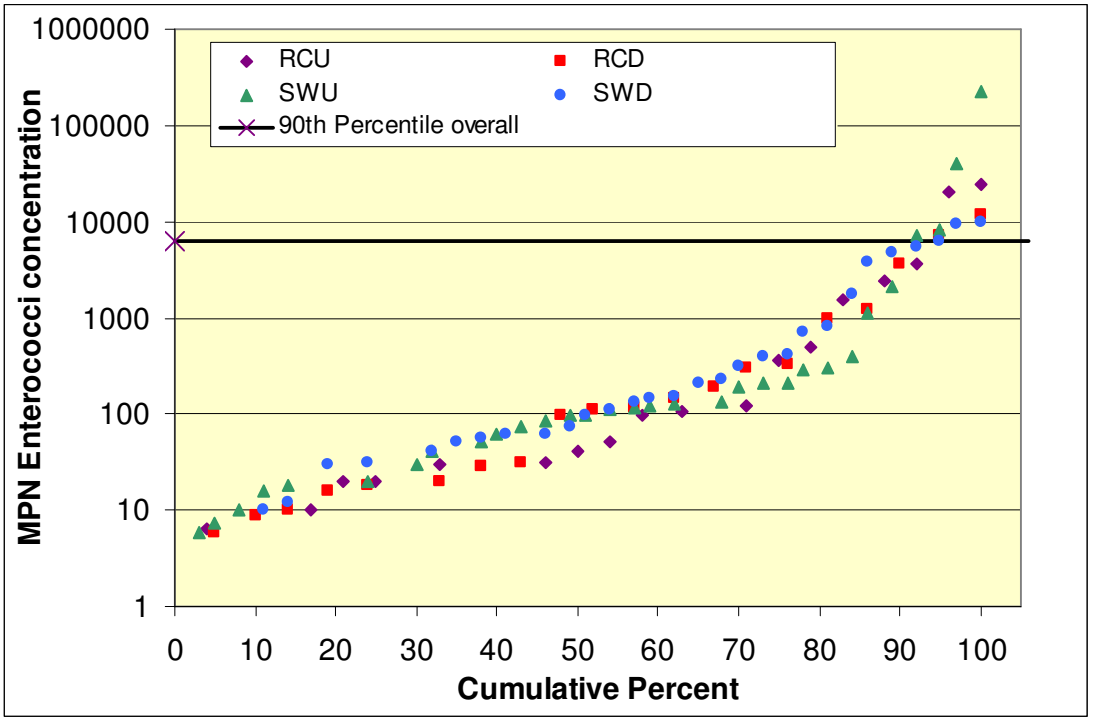
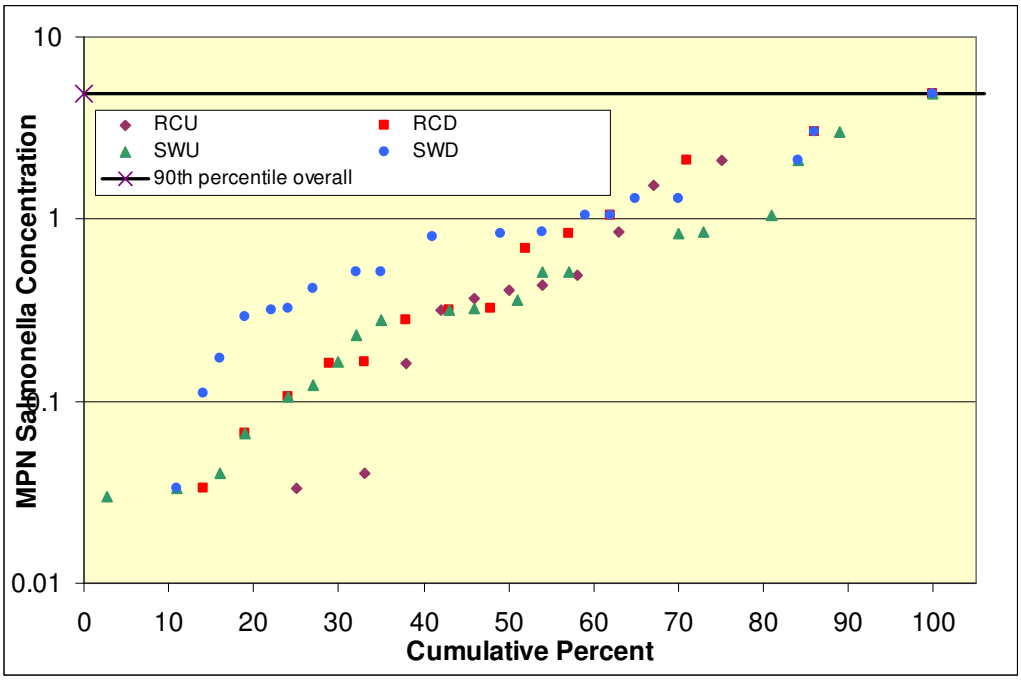


Figure 4.12: Concentration of *Salmonella* in Water Samples by Sample Type as a Function of Cumulative Percent Compared with the Overall 90th Percentile



The number of samples by sample sites for which concentrations are higher than the 90th percentile range from 1 to 6. For the E. coli and Enterococcus sp. indicator bacteria, there are a lower number of samples among the CAFO downstream samples that exceed the 90th percentile than upstream. For *Salmonella*, there were a higher number of samples above the 90th percentile in downstream water samples of CAFOs than those found upstream (table 4. 13). However as there are few samples per group above the 90th percentile conclusive difference cannot be established.

The maximum values in each sample type were also recorded. The maximum concentration of the indicator species is highest in the upstream samples of CAFOs and row crop farms. This is indicative of times when background levels of bacteria are very high. As a result, it is possible that some of the effects of the bacterial contributions by

farms are masked by the high background levels. Furthermore, it is important to note that for *Salmonella*, the 90th percentile is the upper detection limit (4.87 cfu/100ml). As the actual concentration is not known, it is possible that there are even higher concentrations in one or more groups that may be significant. The fact that there are more samples down stream of CAFOs than upstream of the facilities with high concentrations of *Salmonella* could be indicative of contamination of the surface waters by these facilities. However, further studies need to be conducted to elucidate this possible effect if there is any.

Table 4.13: Number of Samples in each Type of Sampling Site for which Bacterial Concentrations Exceeded the Overall 90th Percentile of the Concentrations

Organism	Sampling Site	Number of samples ≥90th percentile	Maximum concentration (MPN)
E. coli	Row Crop Upstream	3	24200 cfu/100ml
	Row Crop Downstream	1	5170 cfu/100ml
	CAFO Upstream	5	125000 cfu/100ml
	CAFO downstream	2	13000 cfu/100ml
Enterococci	Row Crop Upstream	2	24200 cfu/100ml
	Row Crop Downstream	2	12000 cfu/100ml
	CAFO Upstream	4	22800 cfu/100ml
	CAFO downstream	3	9800 cfu/100ml
Salmonella	Row Crop Upstream	6	>4.87 cfu/100ml
	Row Crop Downstream	3	>4.87 cfu/100ml
	CAFO Upstream	3	>4.87 cfu/100ml
	CAFO downstream	5	>4.87 cfu/100ml

Summary

Overall, there were few differences in the bacterial concentrations found in stream water with regard to sample site. In paired analyses, there were no statistical differences in the $\log_{10}$ concentrations of fecal indicators found upstream or downstream of the farms. For *Salmonella* there was a small effect seen, with concentration found upstream lower than downstream. However, this difference is questionable as the samples did not

meet the normality assumption. Further research is required to better elucidate this possible difference. However, given the potential for human health effects associated with exposure to even low levels of *Salmonella*, this potential farm contribution to environmental waters should not be disregarded.

Comparing farm types (swine CAFOs to row crop farms) there were no statistically significant differences found downstream of these farm types. By all analyses, these water samples had similar bacterial concentrations. It is important to note however, that in many cases the water flowing into (or adjacent to) the farms had relatively high concentration of bacteria. This may have resulted in a masking of any true differences in farm impacts on surface water between the two types of farms.

Seasonal Effects

In addition to understanding overall impacts of the farms on bacteria concentrations in water samples or other environmental conditions (relative humidity, air and water temperature), it was also important to understand potential seasonal effects. Each farm, CAFO or row crop, was sampled a minimum of three times in a calendar year. Ambient air temperature and relative humidity were monitored for each sample to identify any anomalies in the expected seasonal weather at the time of sampling. Furthermore the temperature of each sample at the time of sampling was measured and recorded.

Relative Humidity

Overall, season had little effect on relative humidity. While there was a general trend that the warmer the season the higher the relative humidity, there was no significant

difference in relative humidity among seasons (prob >F = 0.1654) based upon ANOVA analyses (table 4.14). This was due in part to the considerable inter-seasonal variability of relative humidity, with minima and maxima differing by about 50 to 60% relative humidity.

Table 4.14: Mean % Relative Humidity on Sampling Days by Season

Season	n	Mean Rel. Humidity	Standard Deviation	Minimum	Maximum
Cool	10	45.3	24.7	18.9	78.0
Moderate	11	50.6	15.5	30.7	87.5
Warm	14	60.4	17.5	36.3	90.5
Overall	35	53.0	19.8	18.9	90.5

Air Temperature

The difference in average air temperature by season was approximately ten degrees Celsius (table 4.15), with an annual maximum of 30.9 ºC), a minimum 11 ºC and an annual seasonal difference of about 20 ºC, overall. The statistical difference by season was analyzed using ANOVA for a one way analysis of variance and probability less than 0.05 is considered significant. A statistically significant difference (Prob > F = 0.0000) was seen in air temperature by season, and there was a relatively strong correlation between air temperature and season (R^2 =0.76), as would be expected.

Table 4.15: Mean Ambient Air Temperature (ºC) on Sampling Days by Season

Season	n	Mean Air Temperature	Standard Deviation	Minimum Temperature	Maximum Temperature
Cool	10	11.0	4.8	3.3	17.5
Moderate	11	21.9	4.7	15.9	29.8
Warm	14	30.9	4.8	24.8	39.6
Overall	35	22.4	9.5	3.3	39.6

Sample Temperature

At the time of sampling, the temperature of each sample was measured; with the exception of cow manure samples. As seen in the air temperature, sample temperatures varied by season. (Figures 4.13 a&b).

Figure 4.13a: Sample Temperature (ºC) by Season in the Various Water Samples

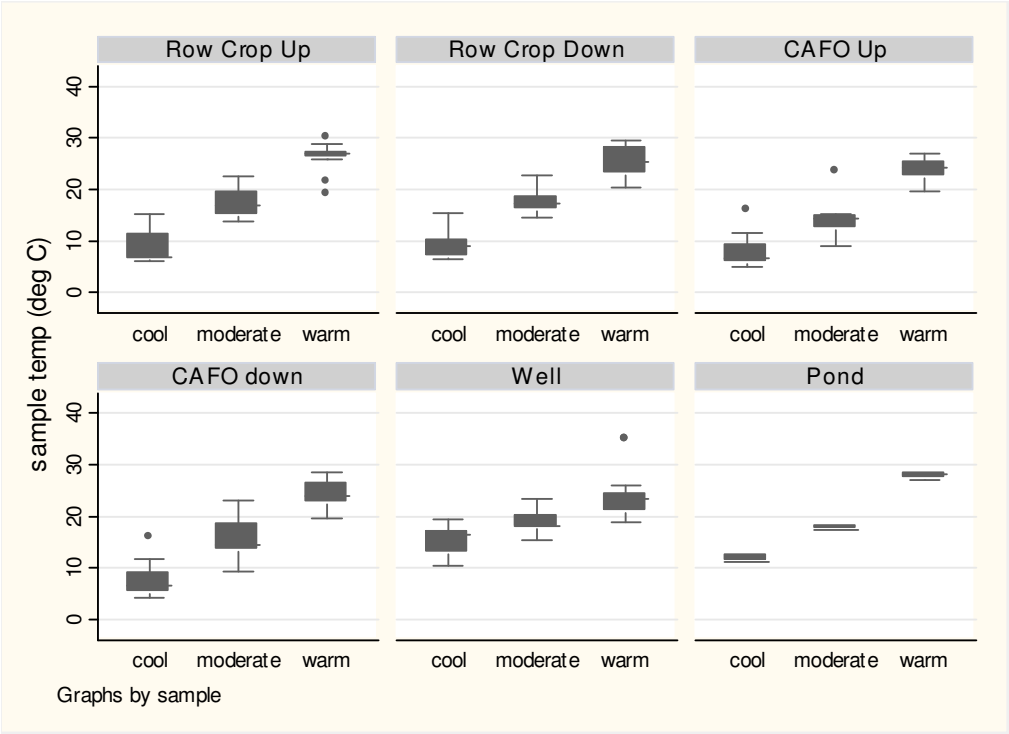
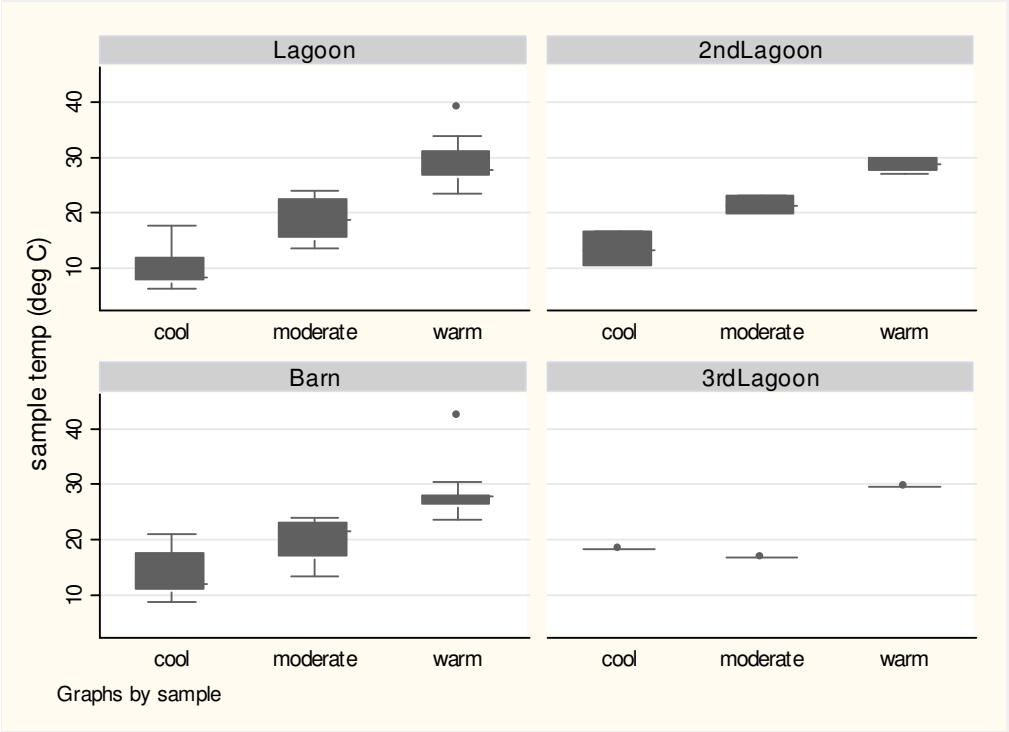


Figure 4.13b: Sample Temperature (ºC) by Season in the Various Swine Waste Samples



As would be expected, the warmer the season, the higher the sample temperature. However, there was some variation in the seasonal differences among the different types of samples. In general, those samples that had less exposure to environmental conditions, such as barn samples and well water samples had slightly lower correlation coefficients than those samples that were more exposed to ambient environmental conditions such as surface water samples and waste lagoons (table 4.15).

Non- parametric analyses were conducted to assess differences in sample temperature by season. The Kruksal-Wallis analysis of variance rank test was used. This test is based upon a χ^2 statistic. As with the ANOVA analysis the level of significance is p= 0.05. In

all samples, there was a statistically significant difference in sample temperature by season.

Table 4.15 Effect of Season on Sample Temperature: Kruksal-Wallis Rank Test Probabilities of Seasonal Differences in Sample Temperature

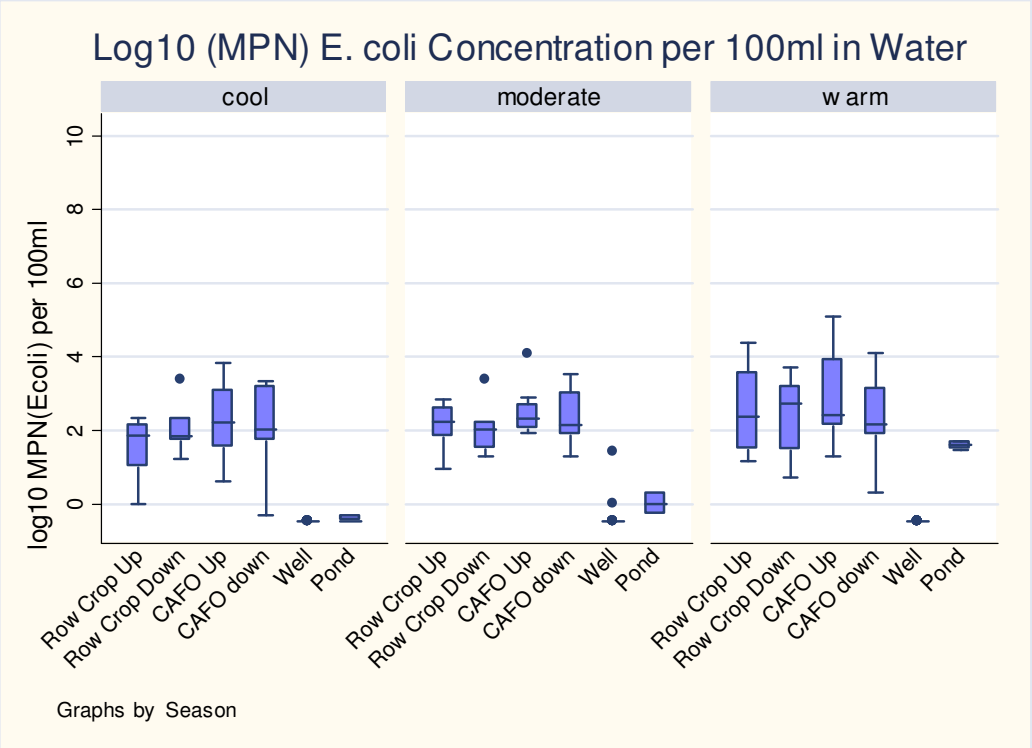
Sample	P	R^2
Stream Water (all)	0.0001	0.8961
Pond	0.0273	0.9844
Well	0.0001	0.7196
Lagoons (all together)	0.0001	0.9049
Barn	0.0001	0.8288

Bacterial Concentrations in Water and Waste

Temperature has been previously reported have an effect on the survival of bacteria in the environment. In this study significant differences were seen in ambient air temperatures and the temperatures of the samples taken, which may have influenced pathogen survival and occurrence. Therefore, bacterial concentrations were analyzed by season to see if there were any significant differences. In these analyses, all stream water samples were pooled for all farms (row crop and CAFO) as well as site (up or downstream) to get the overall seasonal effect on bacteria in water. Furthermore, waste samples were pooled over all farms.

E. coli - In water samples the concentrations of *E. coli* did vary by season (figure 4.14). In stream water the geometric means were somewhat higher in the warmer seasons. The geometric mean concentrations as $\log_{10}$ cfu/100 ml were 2.0 in the cool season, 2.3 in the moderate season and 2.5 in the warm season. In the pond samples, the geometric mean concentrations were -0.4 $\log_{10}$ cfu/100ml, 0.005 $\log_{10}$ cfu/100ml, and 1.6 $\log_{10}$ cfu/100ml in the cool, moderate and warm seasons, respectively.

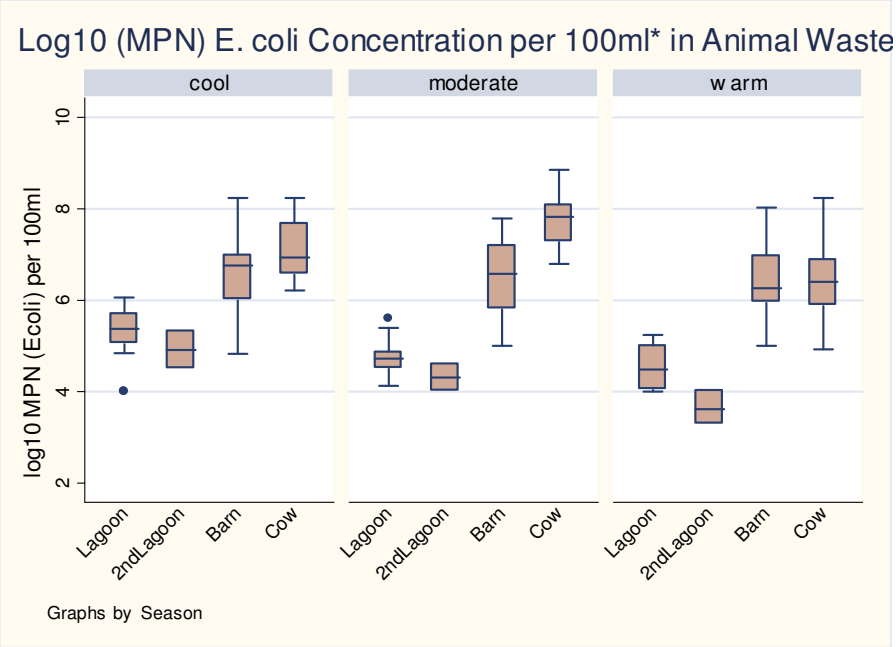
Figure 4.14: Log_{10} *E. coli* Concentrations per 100ml in Environmental Water Samples by Season



Using the Kruskal –Wallis one way analysis of variance rank test, these differences by season were tested for significance differences. This test was used because, as with the sample temperature, the assumptions necessary for using parametric ANOVA analysis were not met, However, as with the analyses of other environmental variables, the level of significance was set at p=0.05. In this analyses it was determined that the seasonal differences in *E. coli* concentrations in stream water were not significantly different (p = 0.1296) but the differences by season in the irrigation pond were statistically significant (p = 0.0312).

As with the water samples, *E. coli* concentrations in waste samples also had some variation by Season (figure 4.15).

Figure 4.15: Log_{10} E. coli Concentrations per 100ml in Animal Waste Samples



* Cow Manure Samples are per gram feces while all others are per 100ml liquid

Analyzing the log_{10}-transformed *E. coli* concentrations by season using the Kruskal-Wallis one way analysis of variance by ranks, it was seen that there was a statistically significant difference in the concentrations found overall, in the primary lagoon samples, and in the cattle manure. However, there were no significant differences in *E. coli* concentration in the barn flush samples or secondary lagoons by season (Table-16).

Table 4.16: Kruskal-Wallis Rank Test Probability Values Comparing *E. coli* Concentrations in Animal Waste Samples by Season

Samples compared	Probability
All Lagoons	0.0005
Primary Lagoon	0.0041
Secondary Lagoon	0.1647
Barn Flush	0.8121
Cow Manure	0.0151

Enterococcus- As with *E. coli* concentrations, there are some seasonal variations among $\log_{10}$ *Enterococcus* concentrations in both water and waste samples (figures 4.16 & 4.17)

Figure 4.16: $\log_{10}$ Enterococcus Concentrations per 100ml in Environmental Water Samples

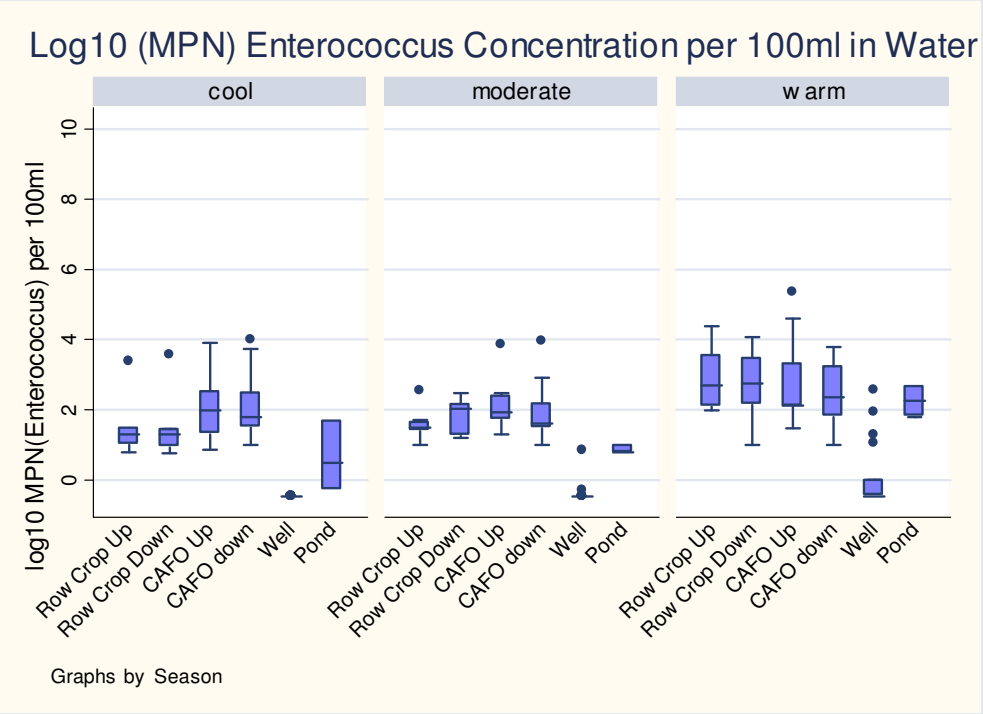
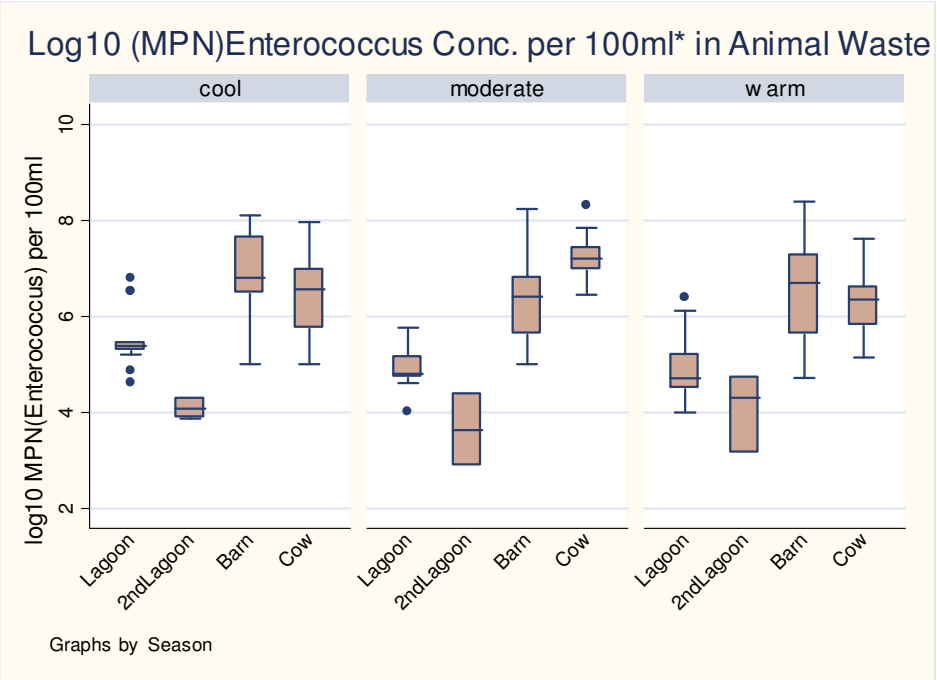


Figure 4.17: Log_{10} Enterococcus Concentrations per 100ml in Animal Waste Samples



* Cow Manure Samples are per gram feces while all others are per 100ml liquid

Analyzing potential seasonal differences in log_{10} *Enterococcus* concentrations using the Kruksal- Wallis test, it was determined that there were seasonal differences in stream samples (pooled), overall lagoon samples, primary lagoon samples and in cow manure. However, no seasonal differences were found in *Enterococcus* concentrations water pond samples or barn flush samples (Table 4.17).

Table 4.17: Kruskal-Wallis Rank Test Probability Values Comparing *Enterococcus* Concentrations in Environmental Surface Water and Animal Waste Samples by Season

Samples compared	Probability
Stream Water	0.0001
Ponds	0.0608
All Lagoons	0.0487
Primary Lagoon	0.0050
Secondary Lagoon	0.8630
Barn Flush	0.5577
Cow Manure	0.0128

Salmonella- There is less seasonal variability in the $\log_{10}$ *Salmonella* concentrations in animal waste and surface water than that associated with concentrations in the fecal indicator bacteria (figures 4.18& 4.19).

4.18: Log_{10} *Salmonella* Concentrations per 100ml in Environmental Water Samples

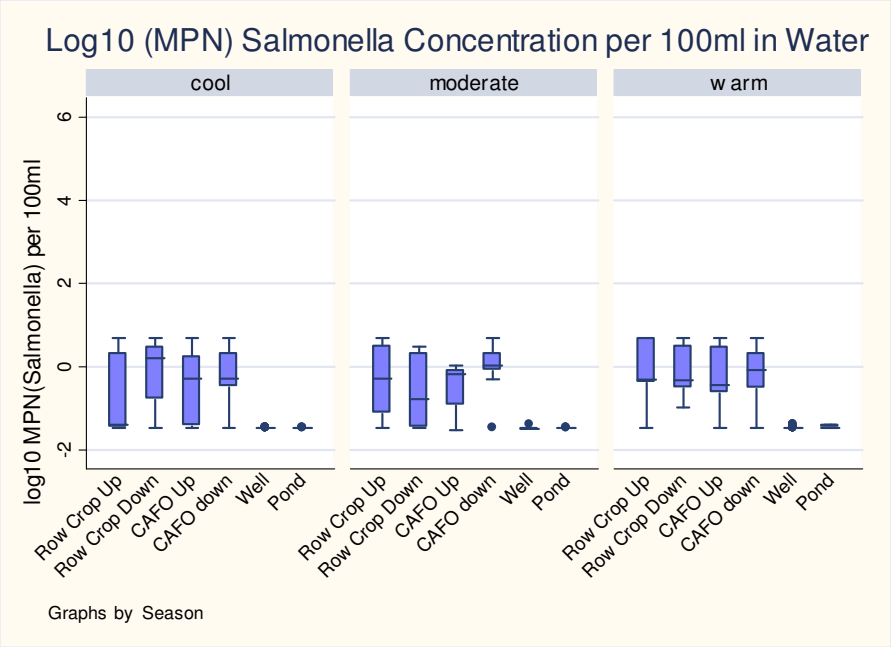
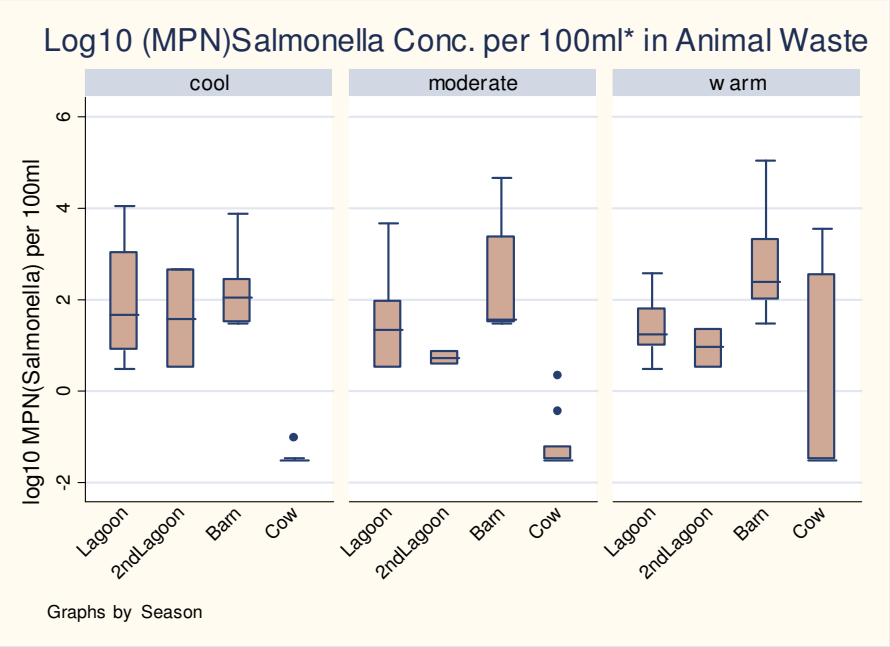


Figure 4.19: Log_{10} *Salmonella* Concentrations per 100ml in Animal Waste Samples



*Cow Manure Samples are per gram feces while all others are per 100ml liquid

Using the Kruskal-Wallis one way analysis of variance test, there were no statistical differences found in log_{10} *Salmonella* concentrations in stream water by season, in surface water samples (stream water p = 0.5817; pond p = 0.7408). Also, there were no seasonal differences in the *Salmonella* concentrations in animal waste samples.

Table 4.18: Kruskal-Wallis Rank Test Probability Values Comparing S*almonella* Concentrations in Animal Waste Samples by Season

Samples compared	Probability
Stream Water (pooled)	05817
Ponds	0.7408
All Lagoons	0.4272
Primary Lagoon	0.4581
Secondary Lagoon	0.152
Barn Flush	0.1480
Cow Manure	0.6920

Summary

Overall there was no seasonal variability associated with *Salmonella* concentration in either water or waste samples. There was some seasonal variation associated with the fecal indicator bacteria. *Enterococcus* concentrations in stream water were statistically higher in the warm season as compared with the moderate and cool seasons. There were no statistical differences *E. coli* concentrations in stream water but in the pond samples concentrations did statistically vary by season. In animal waste samples, there was some seasonal variation in concentration of Enterococci and *E. coli* but the differences were only significant among the lagoon samples and the cow manure samples.

Comparison of Downstream Samples by Farm Type and Season

The above analyses assessed the potential for season to effect bacterial concentrations in the pooled water samples. Additional comparisons that should be addressed are the effect of farm type (i.e. swine CAFO or row crop farm) AND season on bacteria concentrations found in water. In these analyses, multivariate linear regression was used to determine any differences in bacterial concentrations by season and farm. The farm variable was a binomial variable coded 1 for CAFOs and 0 for row crop farms. The season variable was a nominal variable that was coded as indicator variables with the cool season as the referent; e.g. indicator 1 is a binomial variable in which the moderate season is 1 and all others are 0 and indicator2 becomes a binomial variable comparing the warm season, coded 1 to the others coded 0. The model for the assessment is:

Prob (bacterial conc) = α+ β_1 (farm type) + β_2(indicator1 season) + β_3(indicator 2 season) **eqn 4-5**

In these analyses there was only one comparison for which a difference in the downstream sample by season was seen. Log_{10} *Enterococcus* concentrations in the warm season were statistically higher downstream of CAFOs that those found downstream of row crop farms in the same season (p =0.019). In comparisons of all other bacteria and seasons, there were no statistically significant differences.

Bacterial Identification

Archived bacterial isolates were purified and biochemically confirmed. The presumptive *E. coli* and *Salmonella* were biochemically confirmed using Enterotubes™ while the presumptive Enterococcus isolates were identified using APIstrep™ strips. 1390 of the archived bacterial isolates were purified and biochemically tested. This represented 43% of the total isolate library.

488 presumptive *E. coli* were biochemically tested. Of these 458 (94%) were confirmed as *E. coli*. Of the other 30, twelve were confirmed to be some other species (including the most common *Klebsiella pneumonia*), while eighteen had unknown identification codes.

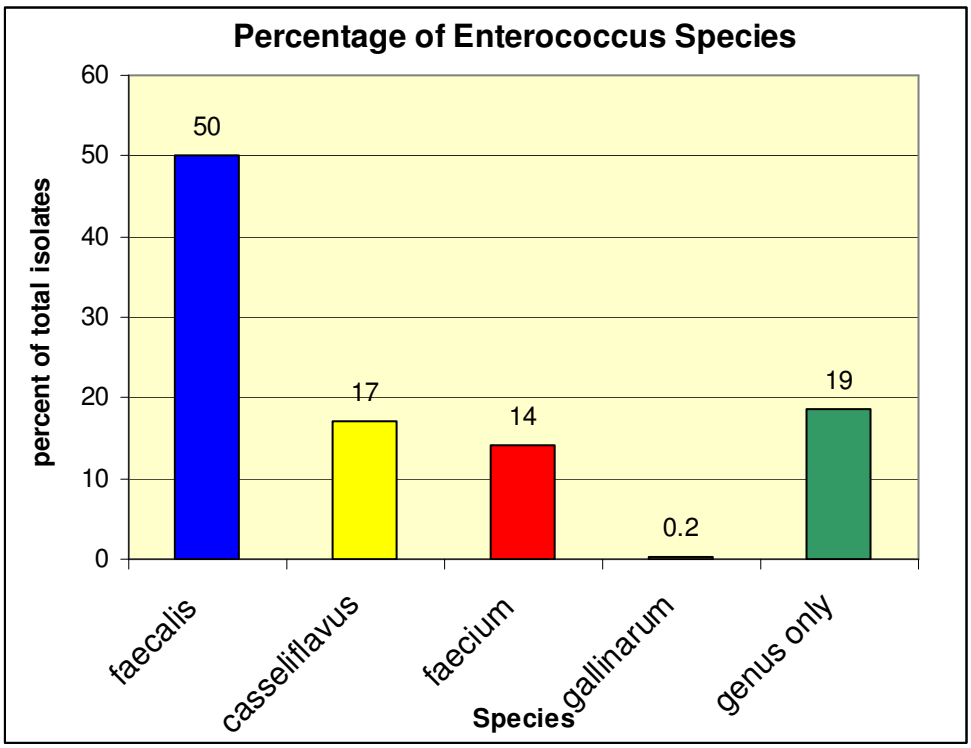
400 presumptive *Salmonella* were biochemically analyzed. Of these 270 (67.5%) were confirmed *Salmonella* sp. Of the remaining isolates, 103 were confirmed as another species (60 isolates being *Proteus mirabilis*), while the remaining 27 had unknown identification codes.

The specificity of the *Salmonella* assays is lower than those of the *E. coli* and Enterococcus assays. This could be attributed in part to the need for enrichment steps in the *Salmonella* assay. As concentrations of this pathogen are generally low, it is necessary to enrich samples to detect any *Salmonella* present. Unfortunately, *Salmonella* sp are not the only bacteria that are enriched in this process. Furthermore, using phenotypic versus genotypic methods can create some ambiguity in genus and species identification, as colonies of many different species may look similar on an agar medium isolation plate and therefore, the potential to isolate a non-Salmonella isolate is higher. Finally, due to laboratory incubator resource limitations, the enrichment cultures were incubated at 41ºC rather than the recommended 43 ºC. Therefore, some of the other non-Salmonella species may have out-competed the *Salmonella* in the enrichment steps.

491 presumptive Enterococcus isolates were biochemically assessed. Of these, 470 or 95% were confirmed as Enterococcus. Of the remainder, 13 were confirmed to be some other species (*Aerococcus viridans* and *Lactococcus lactis* being the most common alternatives). Seven other isolates were possibly Enterococcus but this identification was of low discrimination with regard to other species. One isolate yielded a profile that was inconclusive for the identification of any species.

There were several species of Enterococcus found in the environmental samples (figure 4.20). The most predominant species found was *E. faecalis*, which accounted for 50% of the total Enterococcus species identified. *E. faecium* and *E. casseliflavus* accounted 14% and 17% of the isolates, respectively. 19% of the isolates could only be confirmed to the genus level. The possible species for these isolates included *E. faecalis, E. faecium, E. durans, E. avium,* and *E. gallinarum.*

Figure 4.20: Enterococcus Species Found in Environmental Species



The high prevalence of *E. faecalis* and *E. faecium* was expected as these are predominant human enteric Enterococcus species.(Aarestrup et al (2002)) However, the high prevalence of *E. casseliflavus* was unexpected, as this species has predominantly been known as an environmental Enterococcus. It, along with other yellow pigmented Enterococcus such as E. *mundtii* and *E. sulfurous*, are thought to be primarily plant associated. While these species have been seen in the gut flora of mammals, including people, cattle and poultry, and in insects, it is believed that this species is more transient rather than causing long-term colonization in these animals (Aarestrup et al., (2002), Gelsomino, R. et al. (2003)).

The high prevalence of *E. casseliflavus* may be of public health concern, as this species of Enterococcus is intrinsically resistant to vancomycin. Therefore, if human

exposure occurs and colonization or infection results, this could have a negative impact with regard to both human and animal health and treatment practices.

Overall Summary and Conclusions

Fecal bacteria, including the pathogen *Salmonella* and the resident micro flora of E. coli and Enterococcus sp., were present in high concentrations in animal waste samples on swine CAFOs in eastern North Carolina. There is some reduction in the concentration of these bacteria by treatment in waste lagoons. However, the concentrations still remaining are high enough to have the potential to cause negative human health effects to those who are exposed.

These same bacteria have been found in surface waters surrounding these animal agriculture facilities as well as ambient waters surrounding row crop farms in the region. Overall, however, there seems to be little discernable impact of the farms on the bacteria concentration found in surface and ground waters. Comparing up and downstream samples collected at each site, the overall mean of the differences in $\log_{10}$ concentrations were considered not significant in any of the comparisons. However, when analyzing *Salmonella*, up and downstream of CAFOs, the p value was 0.0516, which is barely significant. While this indicated that there is a not quite significant difference at an α-level of 0.05, it is very close to being significant. Furthermore, as *Salmonella* are true pathogen, any potential source of these bacteria should be taken seriously. Therefore, further examination of these farms as the source of *Salmonella* in water is warranted.

Comparing bacterial concentrations in ambient waters surround row crop farms to those potentially impacted by swine CAFOs, there were not significant differences in the

concentrations of any of the bacterial species. Using several different methods, it was concluded that $\log_{10}$ concentrations of *E. coli*, enterococci and *Salmonella* are similar downstream of each of these farm types.

These findings are similar to those found in studies in other geographic regions. Johnson, J.Y., et al. (2003) conducted a study of bacteria in water in an Alberta, Canada watershed. This watershed consisted of distinct areas which had little impact at all, domestic animal impacts and human impacts. During this study it was found that there was no correlation of manure production and CAFOs and *Salmonella* concentrations in the surface water. In a two year analysis of multiple watersheds in the Pacific Northwest, it was found that while cows grazing in the area did contribute to the bacterial load in the streams, the overwhelming majority (> 84% in 2003 and > 73% in 2004) of fecal contamination was attributed to wildlife (Meays, C.L., 2006). In a watershed with predominately agricultural use in the finger-lakes region, wildlife, specifically geese and deer, were again seen as the major fecal bacteria contributors to the surface waters sampled (Somarelli, J.A., 2007).

While few differences in bacteria concentrations in water were found between farm types, there were some differences seen in bacterial concentrations among farms, i.e. the concentrations of bacteria in water surrounding different farm locations differed. Part of this could be attributed to differences in Enterococcus and *E. coli* concentrations in the waste at the different farm sites. However, it may also be indicative of geographic differences with regard to weather, land use etc. While the all the farms came from a single region, and were relatively similar, there are some geographic and demographic differences that could contribute different bacterial loads. Some factors leading to these

differences could include differences in both point and non-point sources of fecal contamination.

While all the farms in the study are considered to be in rural areas, the housing density around each farm varies to some degree. Not only does this effect the human contribution to the bacterial load itself, but it affects surface permeability. (Mallin, M.A. et al., 2000). For example, the more homes and/or paved roads and driveways reduce surface permeability and more runoff from these areas has the potential to enter the surface waters in the area. Furthermore, some of the study areas rely on septic systems for human waste disposal, while others have community sewers and waste treatment facilities. With the community sewer systems, failures, sewer overflows and storm water intrusion into the sewers would lead to potential point source and non-point source discharges. Failures of septic systems are generally smaller scale, but they can go undetected for long periods of time and can have significant impacts on ground and surface water quality, especially in areas with very porous soils and/or high water tables (Ahmed, E. et al. (2005), Paul J.H. (2005), Scandura, J.E. and Sobsey, M.D. (1997), Yates, M. (1995)).

Another potential factor that could contribute to bacterial concentrations to surface water is the different wild and domestic animal species including birds, reptiles, and rodents, deer, horses and household pets that are found in different geographic areas. Studies have shown that these non-point sources can have a significant impact on the concentrations of both known pathogens, such as *Salmonella* and *Campylobacter,* as well as bacterial fecal indicators in marine, estuarine and fresh water environments (Alderisio, K.A. et al (1999), Alm, E. W. (2003), Anderson, S.A. et al., (1997), Hagedorn, C. et al.

(1999), Levesque, B. et al. (1993), Mallin, M.A. (2000), Meyer, K.J. (2005), Mundt, J.O. (1963)).

Given that bacteria concentrations were generally similar in most stream water samples, it cannot be concluded that the swine CAFOs in this study were major contributors to the measured bacterial concentrations in these surface waters. While these facilities are undeniably a potential source of fecal contamination, with high concentrations of fecal bacteria, including pathogens, present on the farm and in the untreated and treated (lagoon) waste, it does not appear that these bacteria were demonstrably entering surface waters or ground waters, as measured by detectable increases in ambient waters downstream from the farms..

Other studies however, have seen impacts on water quality from large scale animal agriculture facilities (Hooda, P.S. (2000)). The differences could be the result of several factors. First, many of the studies that linked fecal contamination to animal agriculture are older studies. With the awareness of water pollution and its impacts, agricultural activities could have been altered to reduce of prevent contamination of water sources. Many waste management practices have been put in place to reduce nitrogen contamination of the surface waters, and these activities may have also had an effect on fecal contamination. Such activities include timing of spray field irrigation to reduce the likelihood of runoff into surface and ground water, and creating vegetative buffers between the farms and the water source to increase "filtration" of runoff water. As the farms involved in this study were independent family growers, it is likely that any known advances that were feasibly possible would have been employed, as these farmers not only work in the area but also live their as well.

Many of the previous studies on microbial impacts on ambient water have focused on cattle feedlots and grazing fields. While most of the swine CAFOs in this study did graze cattle, they were in relatively small numbers. As a result, even those animals that had direct access to the surface waters sampled may have had limited impact on fecal bacterial loads.

The proximity of the row crop sites to swine animal agriculture facilities (including study and non-study swine CAFOs) was also a potential reason for the lack of impact seen. It is possible that the high swine CAFO densities in some areas resulted in sufficiently high background concentrations of bacteria in the surfaces waters that any affect by the farms in this study were masked. However, while many of the row crop sites in this study were not remote from swine facilities, this does not appear to be a confounder in the overall concentrations. Statistical analysis revealed that there were no significant differences in the bacterial concentrations found in streams between the two kinds of farm sites, swine farms and row crop farms. As some of these farms were completely remote from or strictly upstream from the swine CAFOs, this lack of difference in observed bacteria concentration in ambient waters would indicate that there was in fact, little impact from the non- study CAFOs near row crop sampling sites.

An additional concern with regard to the row crop farms is the use of animal manure for fertilizers on this farm type. This was addressed by monitoring the timing of land application of manure to the farm. If land application had occurred within one month prior to sampling, the soil from the fields was to be sampled as well. At no time during the study did this occur. While there are possibilities for longer term survival of fecal bacteria in the soil environment, this impact was not directly addressed in this study.

It is also important to note that most of the sampling in this study was done under "normal" weather conditions and not those of unusually heavy rain, tropical storm or hurricane conditions. The sampling that was done after extremely heavy rain resulted in higher bacterial concentrations of both fecal indicators and *Salmonella* in all stream water samples. Therefore, during unusual weather events, these facilities could become a major source of contamination.

The above analyses focus on comparisons of bacterial concentrations in stream water. However, examination of this type of environmental sample is not sufficient to determine the actual sources and possible pathways of fecal contamination. Furthermore, analysis of bacteria concentrations alone does not address the human health impacts possibly created by swine waste sources getting into ambient waters. For this reason, antibiotic resistant patterns of the bacteria found in environmental samples were examined to further characterize any potential risks of bacteria originating from the swine animal agriculture facilities of this study.

Chapter 5 – Antibiotic Resistance Analysis

While enteric bacterial concentrations in the environment can be an indication of fecal contamination from various sources, some other traits of the bacteria present are important to public health. If the bacterium is pathogenic, such as *Salmonella*, it is of greater concern to public health than non-pathogenic bacteria. But pathogenicity is not the only property that can lead to public health concerns. Resistance traits that help the bacteria survive or compete are also of concern. While there are other substances to which bacteria can be resistant, such as heavy metals, in this research the focus was on antibiotic resistance.

Antibiotic resistant bacteria are of growing concern worldwide. While not all antibiotic resistant bacteria cause human illness, they have the potential to spread resistance genes to other bacteria. Opportunistic bacteria are of special concern because these relatively harmless bacteria that infect or colonize hosts now pose greater risk of persisting due to their inability to be eliminated by antibiotic therapy. Hence, antibiotic resistance creates potential human health risks, even from opportunistic or colonizing bacteria. As a result of these concerns, there has been an effort to identify the various sources of resistant bacteria and reduce antibiotic usage when possible.

The use of antibiotics in animal agriculture at sub-therapeutic levels has been of particular concern. Animals receiving sub-therapeutic doses of bacteria can develop intestinal bacterial flora with high levels of resistance, and these bacteria are fecally shed

at levels and are readily detectable in untreated and even treated animal agriculture waste. Little research has been done to examine the actual impact of antibiotic usage in animal agriculture and the excreted antibiotic resistant bacteria on the environment and communities surrounding the farms where these bacteria originate.

In this research, animal agriculture facilities (or Confined Animal Feeding Operations (CAFOs)) were assessed for their potential impact on their surroundings with regard to antibiotic resistant bacteria. Analyses were conducted to determine 1) if, and to what extent, there are antibiotic resistant bacteria present in animal wastes on swine CAFOs; 2) if those bacteria are released into environmental water, including ground and surface water; and 3) if people who live near or work on CAFOs are exposed to, and consequently acquire, resistant bacteria through environmental water as a result of their association with these facilities.

E. coli, *Salmonella* sp. and *Enterococcus* sp. isolated from animal waste, environmental waters (as described in Chapter 4) and human study participants (Chapter 6) were analyzed for an array of antibiotics. The *E. coli* and *Salmonella* were characterized using a suite of antibiotics important for human and veterinary health targeting Gram-negative bacteria while the enterococci were characterized using a suite of antibiotics relevant to Gram-positive bacteria. The frequency of resistance to individual antibiotics and patterns of multi-drug resistance from each source (e.g. water, animal or human waste samples, farm association etc.) were determined. The resistance patterns were then compared by source to determine differences or similarities.

Materials and Methods

As discussed in Chapter 4, *E. coli*, *Salmonella* and *Enterococcus* isolates were obtained from animal waste samples: lagoons, barn flush and cattle manure; and from the following water samples: ground water wells located on animal agriculture facilities, stream water up and down stream of animal agriculture and non-animal agriculture facilities, and in one case irrigation ponds located throughout a non-animal agriculture farm. As bacterial concentrations were quantified in each of these samples, bacterial isolates of the three target microorganisms were also collected. Up to five isolates per sample collected were archived for further analysis. Of these isolates, the first two of each sample were purified and biochemically confirmed. If one of these isolates was found to be some other species besides the intended target species, then another isolate from the sample (if available) was purified and biochemically tested. Once biochemical confirmation was achieved, these isolates were then further characterized for phenotypic antibiotic resistance traits. A total of 453 environmental *E. coli*, 276 environmental *Salmonella* and 418 environmental *Enterococcus* sp. were tested for antibiotic resistance.

In addition to the environmental isolates, bacterial isolates were also collected from human subjects who agreed to participate in the study. As human subjects were involved, this study was reviewed in advance by the Institutional Review Board (IRB) at Wake Forest University as well as approved by the CDC as they were the funding source. All study participants signed an informed consent form prior to enrollment into the study.

Study participants were asked to submit fecal samples to Wake Forest University Baptist Medical College (WFUBMC) laboratory once a month for a year. This period corresponded with the time during which environmental sampling occurred in their

neighborhood. The submitted fecal samples were analyzed at WFUBMC for *E. coli*, *Enterococci* sp *Salmonella* sp and *Campylobacter*. As *E. coli* and Enterococci are common gastrointestinal bacteria in humans, these bacteria were pre-screened for at least minimal resistance to one of several antibiotics (table 5.1). The isolates that grew in the presence of any of the antibiotics were purified, biochemically identified (using Enterotubes® by Becton Dickenson™ or Api20strep strips® by bioMérieux™, as appropriate) and archived for further analysis, including antibiotic resistance profiles. As *Salmonella* and *Campylobacter* are true pathogens, their presence was a concern in itself. Therefore, they were not prescreened for antibiotic resistance and were to be archived for further analysis, including antibiotic resistance. However, in this study, there were no instances in which *Salmonella* or *Campylobacter* were isolated. From human specimens, there were 148 *E. coli* isolates and 265 Enterococcus isolates that were archived and tested further for antibiotic resistance. All isolates that grew on the selective media were archived. Almost half of the specimens submitted did not have resistant bacteria. Of the remaining specimens, most had only one isolates that grew, however, there were some specimens for which up to 6 isolates were collected. There were specimens that had only *E. coli* or *Enterococci* sp, while others had both of them present.

Table 5.1: Concentrations of Prescreening Antibiotics for Isolation of Human Bacteria

For E. coli:	**For Enterococcus**
ciprofloxacin 2ug/ml	ampicillin 8 ug/ml
gentamicin 4 ug/ml	Gentamicin 250 ug/ml
norfloxacin 4 ug/ml	streptomycin 250 ug/ml
tetracycline 4 ug/ml	Quinupristin/dalphopristin 2 ug/ ml
	Vancomycin 8 ug/ml
	tetracycline 4 ug/ml

Antibiotic Resistance Testing

Antibiotic resistance profiles were determined using Minimum Inhibitory Concentration (MIC) break points as set by the Clinical Laboratory Standards Institute (CLSI) (2002) (formerly NCCLS – National Committee of Clinical Laboratory Standards) and the National Antibiotic Resistance Monitoring System (NARMS) (breakpoints used are outlined in table 5.2). Some of the antibiotics examined were those used exclusively in veterinary medicine rather than in human clinical use, therefore, breakpoints were not established for these antibiotics. In these cases, the MIC_{50} and MIC_{90} values of the bacteria isolated in this study are reported. These values are the minimum concentrations at which 50% and 90% of the isolates analyzed in this study are susceptible to the antibiotic.

Sensititre™ multi-well MIC plates by TREK Diagnostics® were used to determine the antibiotic resistance profiles of the bacterial isolates collected. These are specialized plates that utilize a micro-dilution method for determining antibiotic resistance. These were 96 well plates in which each well contains a different antibiotic and concentration of it. Each antibiotic had a range of concentrations in different wells to establish growth/no growth gradients. The wells were inoculated with a standard concentration of bacteria (10-200 cfu/µL) and after the incubation period they were scored for growth. The highest concentration for each antibiotic at which there is growth was recorded. Using the MIC breakpoints (or in the case of strictly veterinary drugs, MIC_{50} and MIC_{90}), the isolate was determined to be susceptible, intermediate or resistant.

In this research antibiotics that are important to both human and veterinary medicine were of interest. The veterinary plate layouts by TREK Diagnostics® were

designed for such research in collaboration with the US FDA Center for Veterinary Medicine as well as other experts. For this study two of the plate designs were used: CMV1AGNF and CMV1AGPF.

For the Gram-negative bacteria (*E. coli* and *Salmonella*) the CMV1AGNF plate was used. This plate consists of 15 different antibiotics with appropriate ranges (see table 5. 2). It is important to note that for sulfisoxazole, the concentration gradient only reaches 256µg/mL while the resistance breakpoint is 512µg/mL. This is because Trek diagnostics™ is only certified to use up 256µg/mL in the Sensititre® product. Commonly, isolates that are resistant at 256µg/mL are also resistant at 512µg/mL. To confirm this, a subset (about 15%) of the isolates in this study found to grow at 256µg/mL were further tested at 512µg/mL using a macro-broth dilution method. Greater than 95% of the isolates in this subset did, in fact, grow in the presence of 512 µg/ml of sulfisoxazole. The one isolate for which there was no growth, also did not grow at any of the concentrations of sulfisoxazole in the macro-broth dilution test. Furthermore, negative control organisms including four isolates that tested negative for sulfa-resistance in by the Sensititre plate – micro dilution method as well as an ATCC *E. coli* strain that is not sulfa resistant were also tested and in each case, these organisms did not grow at any of the concentrations of sulfisoxazole – confirming that the test itself was reliable. Based on these results, all isolates with positive growth at 256µg/mL by the Sensititre micro- dilution plate method are considered resistant to the drug.

Table 5.2: Gram Negative Plate Antibiotics and Dilutions tested and MIC Breakpoints

Antibiotic	Dilution Range (µg/ml)	Break point		
		Susceptible	Intermediate	Resistant
Amikacin	0.5-64	≤16	32	≥64
Ampicillin	1-32	≤8	16	≥32
Amoxicillin/ Clavulanic Acid (Augmentin™)	1/0.5 – 32/16	≤8/4	16/8	≥32/16
Ceftriaxone	0.25-64	≤8	16-32	≥64
Chloramphenicol	2-32	≤8	16	≥32
Ciprofloxacin	0.015-4	≤1	2	≥4
Trimethoprim/ Sulfamethoxazole	0.12/2.38 -4/76	≤2/38	--	≥4/76
Cefoxitin	0.5-32	≤8	16	≥32
Gentamicin	0.25-16	≤4	8	≥16
Kanamycin	8-64	≤16	32	≥64
Nalidixic Acid	0.5-32	≤16	--	≥32
Sulfisoxazole	16-256*	≤256	--	≥512
Streptomycin	32-64	≤32	--	≥64
Tetracycline	4-32	≤4	8	≥16
Ceftiofur	0.12-8	≤2	4	≥8

* Breakpoint for this drug is 512 µg/ml; further analyses done to confirm resistance at 256 µg/ml was indicative of resistance at 512 µg/ml

For the Gram positive bacteria (*Enterococcus* sp.) the CVM1AGPF plate was used. This plate consisted of 17 different antibiotics (table 5.3). Twelve of these drugs are important for human use and treatment; ten of which are used in the treatment of gram positive infections, including those caused by *Enterococcus* sp. The other two drugs (nitrofurantoin and kanamycin) are included in the panel but are not commonly used for enterococcal infections. Like sulfisoxazole on the Gram-negative plate layout, the concentration range of nitrofurantoin did not reach the MIC breakpoint. However, because this drug is not commonly used for *Enterococcus* sp., no further examination was done on the isolates that were resistant to the highest concentration. Kanamycin is a drug that was used in clinical settings, but because this drug is no longer commonly used to

treat infections, there is no MIC breakpoint established. Therefore, this drug was treated in the same way as a veterinary drug, calculating MIC_{50} and MIC_{90} values as described below.

At the commencement of this study, five antibiotics were used strictly in veterinary settings. As none of these drugs were used in clinical medicine, no breakpoints for resistance were established (since then tigecycline and daptomycin have been used for human used and there are now CLIS breakpoints established). Therefore, for these drugs MIC_{50} and MIC_{90} values were calculated based upon the results from the resistance analysis of the isolates of this study. The MIC_{50} is the concentration at which 50% of the isolates were inhibited and considered the intermediate level of resistance; and those that grew at the MIC_{90} concentration (the concentration at which 90% of the isolates were inhibited) were considered fully resistant. When Analyzing the MIC_{90} values of three of the antibiotics: flavomycin, tylosin tartrate and tigecycline, it was found that more than 10% of the isolates were resistant to the highest concentration tested. Therefore, in this case, the MIC_{50} value is reported, and the MIC_{90} is reported as greater than the highest dilution. With lincomycin, 78% of the isolates tested were resistant to this drug at the highest level tested. Therefore, "greater than" (>) the highest concentration tested is reported for both the MIC_{50} and the MIC_{90} values.

Due to the different ways in which resistance is considered (i.e. breakpoints versus MIC_{90} values), single and multiple antibiotic resistance analyses were conducted in two groups, those with clinical significance and therefore, established breakpoints, and those that are primarily veterinary drugs. The overall comparison of profiles however, combines the two groups of drugs.

Table 5.3: Gram Positive Plate Antibiotics, Dilutions tested and MIC Breakpoints

Antibiotic	Dilution Range (µg/ml)	Break point		
		Susceptible	Intermediate	Resistant
Chloramphenicol	2-32	≤8	16	≥32
Erythromycin	0.5-8	≤0.5	1-4	≥8
Penicillin	0.5-16	≤8	--	≥16
Quinupristin/Dalfopristin (Synercid™)	1-32	≤1	2	≥4
Tetracylcine	4-32	≤4	8	≥16
Vancomycin	0.5-32	≤4	8-16	≥32
Ciprofloxacin	0.12-4	≤1	2	≥4
Linezolid	0.5-8	≤2	4	≥8
Nitrofurantoin	2-64*	≤32	64	≥128
Gentamycin	128-1024	<500	--	≥500
Streptomycin	512-2048	<1000	--	≥1000
			MIC_{50}	**MIC_{90}**
Kanamycin	128-1024		128	>1024
Daptomycin	0.5-16		1	4
Flavomycin	1-16		4	>16
Lincomycin	1-32		>32	>32
Tigecycline	0.015-.5		0.25	0.5
Tylosin Tartate	0.25-32		4	>32

* plate concentration does not reach breakpoint for this antibiotic

Procedures

Sensititre Antibiotic Resistance Profile Test Procedure

The archived, purified and biochemically confirmed bacterial isolates were streaked onto Tryptic Soy Agar (TSA) and incubated 18-24 hours at 37ºC. From these plates 1 to 5 colonies were taken and placed into 4mL sterile lab grade water. The number of colonies needed was dependent on the size of the isolated colonies on the TSA

plate, as a standard concentration was required. The inoculated water was then mixed well by vortexing and compared with a McFarland™ turbidity standard of 0.5. When the inoculated water matched the turbidity standard, 10µL of this inoculum suspension was transferred into 11mL of Cation Adjusted Mueller Hinton broth with TIS (product of Sensititre™). The inoculated broth was well mixed by vortexing and then poured into a sterile 50mL reservoir. Using a multi-channel pipet, 50µL of the broth was added to each of the 96 wells in the plate. The plate was then covered with the provided film and incubated for 18-24 hours (the Gram-positive plates were all 24 hours to confirm the Vancomycin results). Additionally, 1µL was placed on a TSA plate and spread plated with the use of a sterile glass "hockey stick" spreader. This was used to confirm the concentration of the inoculum as well as provide a purity test to assess for contamination or mixed colonies from the source. These positive control plates were also incubated 18 - 24 hours.

After the incubation period, the MIC plates were place on a mirrored apparatus in which the bottom of each well could be easily seen. Each well was examined for any growth and recorded. Even a tiny amount of growth was considered positive in this analysis, except for sulfisoxazole which is generally a bacteriostatic rather than a bacteriocidal antibiotic. In this case any growth greater than 20% of the positive wells was considered positive for growth and therefore evidence of resistance.

Sulfisoxazole Screening

A 5–tube broth dilution system was set up to determine the MIC of presumed sulfisoxazole resistant *E. coli* and *Salmonella*. Each of the five tubes contained 2mL of Cation Adjusted Mueller Hinton Broth with different concentrations of sulfisoxazole: 512µg/mL, 256µg/mL, 128µg/mL , 64µg/mL and broth with no drug. Isolates for this screening were prepared as those for the Sensititre plate analyses: archived isolates were streaked on TSA, incubated 18-24 hours and 1-4 colonies were selected and added to 4mLs sterile lab grade water to achieve turbidity equal to that of the 0.5 McFarland standard. A one to ten dilution of the inoculated water was then made by placing 10µL of inoculum into 90 µL of phosphate buffered saline solution (PBS). Five µL of the diluted inoculum was then added to each of the 5 tubes and incubated for 18-24 hours at 37ºC. After the incubation period, the tubes were examined for turbidity. Because sulfisoxazole is bacteriostatic, the turbidity of each tube in the series was compared with the tube having no drug. The culture was considered positive if the turbidity was at least 20% of that in the tube with no drug. From each tube, 10µL was then streaked onto a TSA plate (with no antibiotics) to ensure bacterial presence and growth in the culture as well as purity of the culture in the tube.

Results

Overview of Antibiotic Resistance in Environmental Isolates

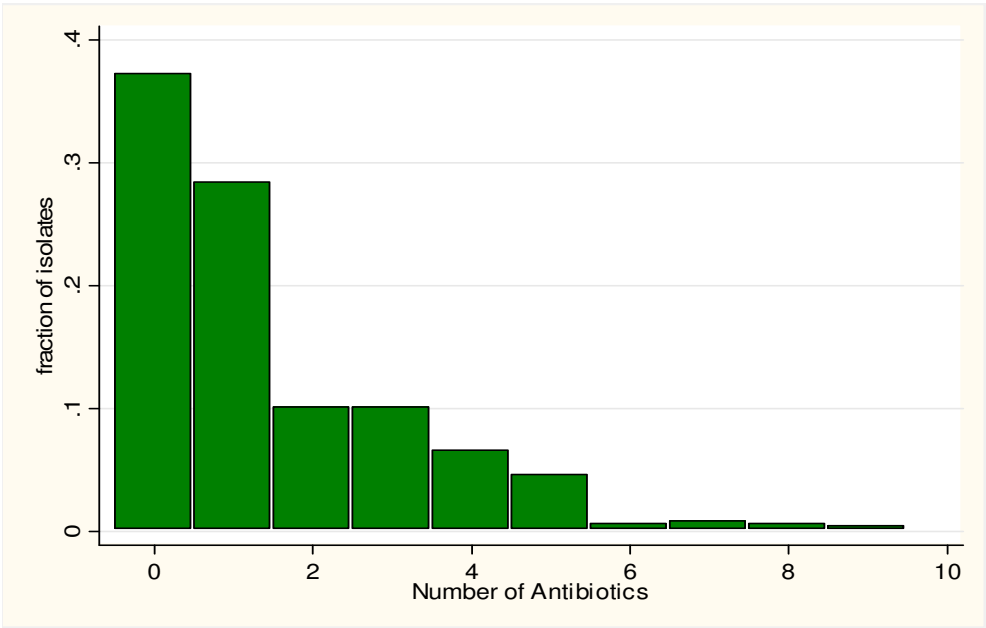
Antibiotic resistant bacteria were found in all sources, human and environmental, analyzed in this study. However, there were environmental isolates that did not have any

resistance traits as well as people that did not harbor resistant bacteria. The number of antibiotics to which the bacteria were resistant varied by species of bacteria, as well as the sample from which they were isolated. In general the swine waste samples had higher proportions of resistant bacteria than any of the other environmental samples. This pattern held true regardless of the genus of bacteria analyzed.

Environmental E. coli - There were 453 *E. coli* isolates collected from environmental samples. Of these, 199 were isolated from stream water samples, 13 from ponds, 4 from ground water wells, 105 from swine lagoon samples, 79 from barn flush samples, and 53 from cattle manure samples.

Of all environmental *E. coli* isolates, 37.3% (169/453) were not resistant to any of the tested antibiotics. Of the 63.7% with antibiotic resistance, 28.5% were resistant to only one antibiotic and the remaining 34.2% were resistant to two or more antibiotics (figure 5.1).

Figure 5.1: Fraction of Environmental E. coli Isolates Resistant to Different Numbers of Antibiotics (n=453)

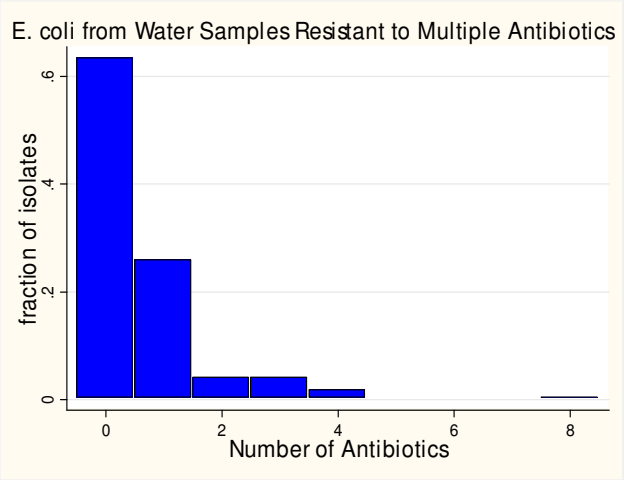


While the overall frequency indicates a minority of (37%) isolates lacking antibiotic resistance, further analyses by source of the bacteria indicates certain sources have a higher proportion of drug resistant isolates than others, which may skew the overall distribution.

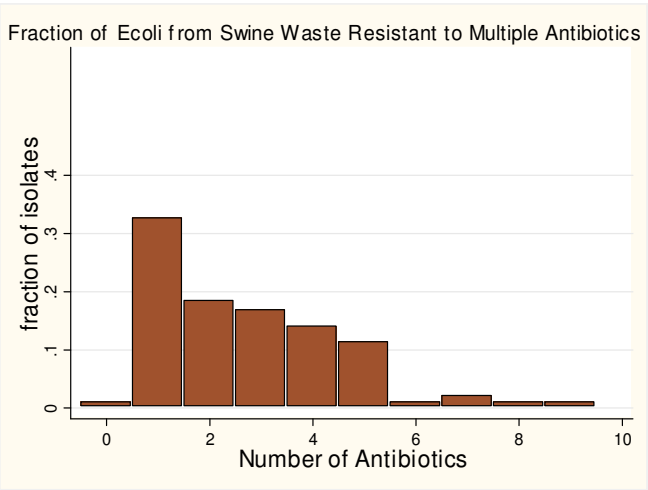
When examining frequency of resistance in isolates by sample type, it is seen that the isolates from water samples including ground and surface water samples, had a much lower frequency of resistant *E. coli* than the frequency of resistant *E. coli* isolates from all sources combined. Of the *E. coli* isolates collected from water samples, 63% had no resistance to antibiotics; 26% were resistant to only one antibiotic; and only 11% were resistant to multiple antibiotics (Figure 5.2). Similar to the water samples, *E. coli* isolated from cattle manure also had a lower frequency of resistance than that of the overall environmental isolates, with 56.6% of the isolates not resistant to any antibiotics, 24.5% resistant to one antibiotic, and only 19% (10 isolates) resistant to 2 or more antibiotics. Isolates collected from swine waste (both barn flush and lagoon samples) however, had the highest frequency of multiple antibiotic resistance. Only 1% (2/184 isolates) were not resistant to any antibiotics and 66% were resistant to 2 or more antibiotics. More than 30% (57/184 isolates) of the isolates from swine waste were resistant to 4 or more antibiotics. By comparison, in water samples less than 3% of isolates (5/216) were resistant to 4 or more antibiotics.

Statistical analyses comparing the frequency distributions of drug resistance in *E. coli* in swine waste and surface waters using the Kolmogrov-Smirnov test and proportion analyses indicate that the frequency of single and multi-drug resistance in swine waste was statistically higher than that found in surface water samples ($p < 0.0001$ for all tests).

Figure 5.2: Frequency of Multiple Antibiotic Resistance in *E. coli* Isolated from Ground and Surface Water (n=216) (a), and Swine Waste (n=184) (b)



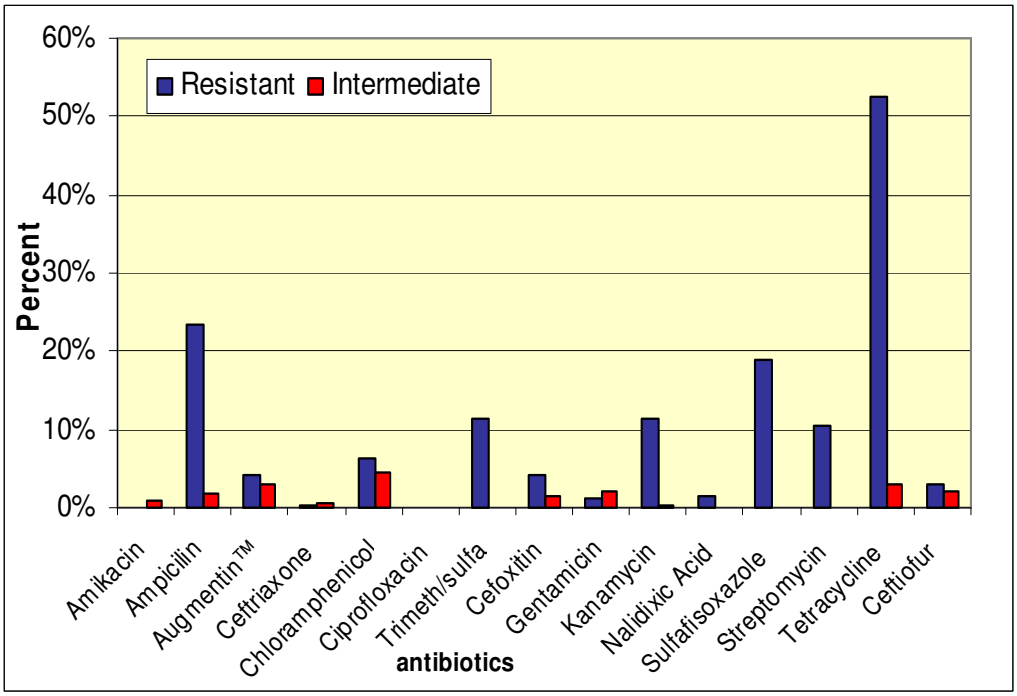
a) Environmental Water Samples



b) Swine Waste Samples

While resistance was high in swine waste, there were no bacteria isolated from waste, or any other sample that were resistant to all of the antibiotics tested. However, of the fifteen antibiotics analyzed, all but two had at least one bacterial isolate with resistance to it. ciprofloxacin and amikacin were the only antibiotics that were effective against all environmental *E. coli* isolates. The antibiotic for which there was the greatest resistance frequency was tetracycline with greater than 50% of all isolates (238/453) testing resistant. Resistance to amplicillin and sulfisoxazole was also prevalent with 23% and 19% of the isolates, respectively, having resistance to them (figure 5.3).

Figure 5.3: Percent of Total Environmental *E. coli* Isolates Resistant to the Various Antibiotics

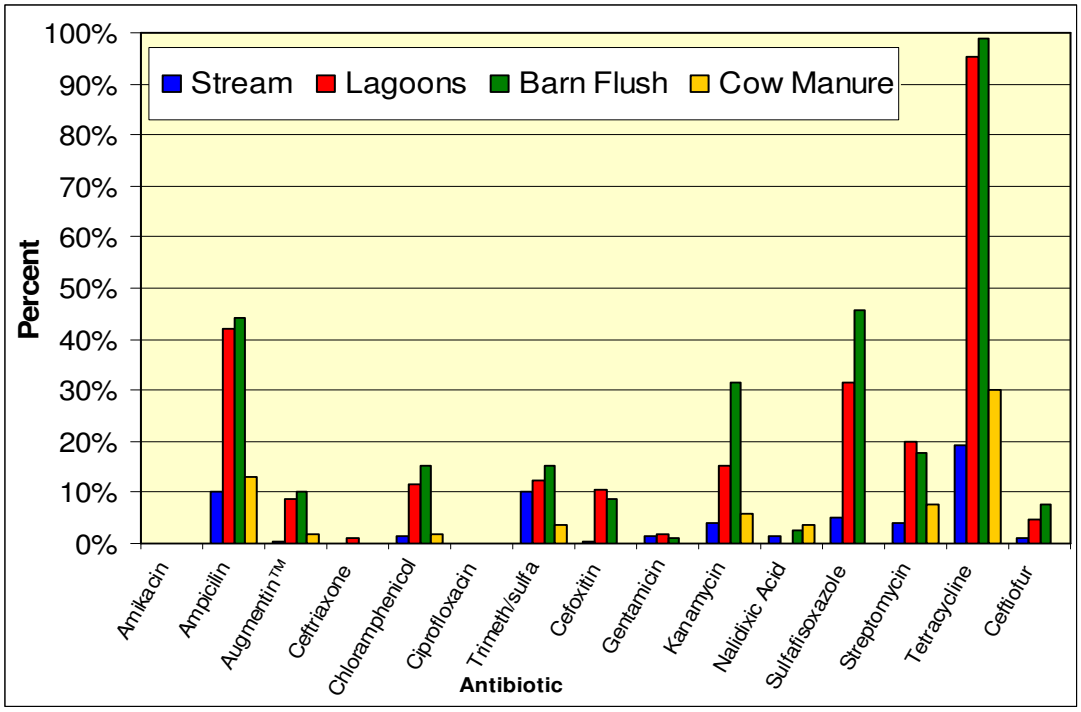


Examining antimicrobial resistance in isolates by sample source (figure 5.4), tetracycline resistance was most frequent in every sample type. As shown in Figure 5.4, nearly 100% of the *E .coli* isolates from swine waste (100/105 isolates from lagoons and 78/79 isolates from barn flush), 30% (16/53) of isolates from cattle manure samples and almost 20% (38/199) of isolates from stream samples were resistant to tetracycline. Furthermore 4 out of 4 *E. coli* isolated from ground water wells were resistant to tetracycline, which was the only drug to which they were resistant.

There were six additional antibiotics to which at least 10% of *E. coli* isolated from swine waste were resistant: ampicillin, kanamycin, chloramphenicol, trimethoprim/sulfamethoxazole, sulfisoxazole and streptomycin. In isolates collected

from stream water, there were no antibiotics other than tetracycline for which greater than 10% of the isolates were resistant.

Figure 5.4: Percent of *E. coli* Isolates by Sample Type Resistant to Studied Antibiotics

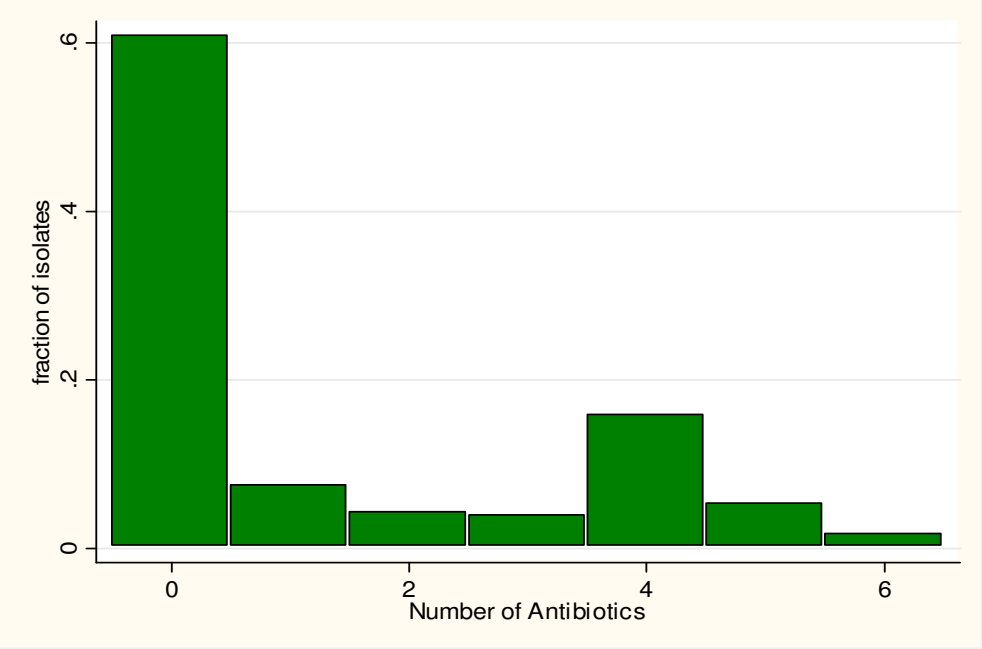


Environmental Salmonella

While not as prevalent as *E. coli*, *Salmonella* sp. were found in most environmental samples (see Chapter 4). A total of 276 *Salmonella* isolates were purified and biochemically confirmed from environmental samples. The sources of these were: 165 isolates from stream water samples, 70 from swine lagoons, 34 from barn flush samples and 5 from cattle manure. No *Salmonella* were found in ground water wells and only 2 isolates were found in the irrigation ponds.

Unlike the *E. coli* isolates, the majority of *Salmonella* isolates tested were not resistant to any antibiotics. 61% of *Salmonella* isolates (168/276 isolates) were found to have no resistance, 7.6% (21/276 isolates) were resistant to one antibiotic and 31% were resistant to multiple antibiotics. Five isolates (1.8%) were resistant to six different antibiotics (figure 5.5). Comparing the proportion of environmental *Salmonella* isolates resistant to at least one antibiotic to the proportion of environmental *E. coli* isolates with the same, it is seen that the two proportions differ significantly ($p < 0.0001$). However, when comparing the proportions of multi-drug resistance (fraction of those isolates resistant to two or more antibiotics) in environmental *E. coli* and *Salmonella* isolates, no significant difference was found ($p = 0.4536$).

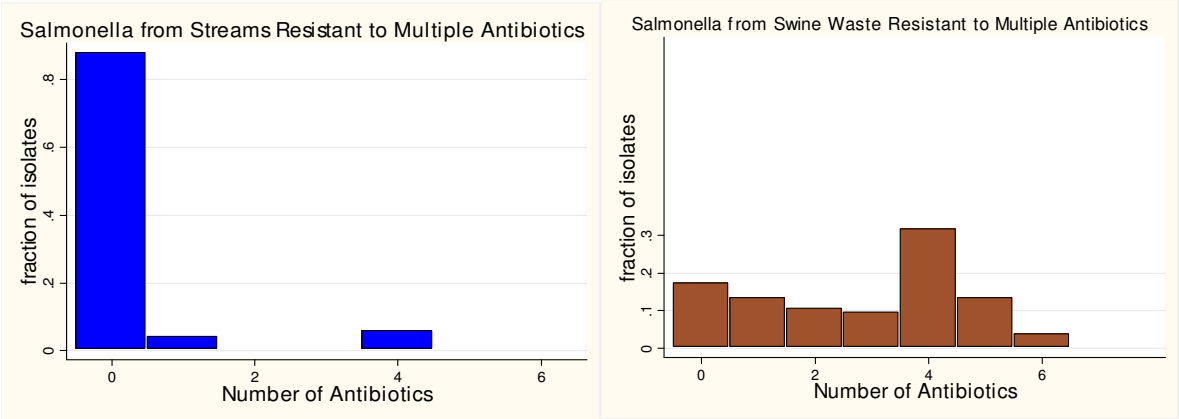
Figure 5.5: Fraction of Environmental *Salmonella* Isolates Resistant to Different Numbers of Antibiotics



Similar to what was found with the environmental *E. coli*, there was a higher frequency of antibiotic resistant *Salmonella* in swine waste samples than in stream water samples. In swine waste samples only 17% of Salmonella lacked resistance to any antibiotics, while 13% were resistant to only one antibiotic and 69% were resistant to 2 or more antibiotics (figure 5. 6b). In contrast, 88% of *Salmonella* isolates from surface water lacked resistance to any antibiotics, 4% were resistant to 1 antibiotic, and 8% were resistant to 2 or more antibiotics (6% resistant to 4 antibiotics and 0.6% (1 isolate) resistant to 5 and 6 antibiotics each) (figure 5.6a).

Comparing the frequency distributions and proportions of single and multi-drug resistance in *Salmonella* in swine waste and water, it was found that there was a significantly higher frequency of resistance in the *Salmonella* isolates collected from swine waste samples than those isolated from water samples ($p < 0.0001$ for all tests).

Figure 5.6: Frequency of Multiple Antibiotic Resistance in *Salmonella* Isolates from Stream Water (a), and Swine Waste (b)



a) Stream Water Samples **b) Swine Waste Samples**

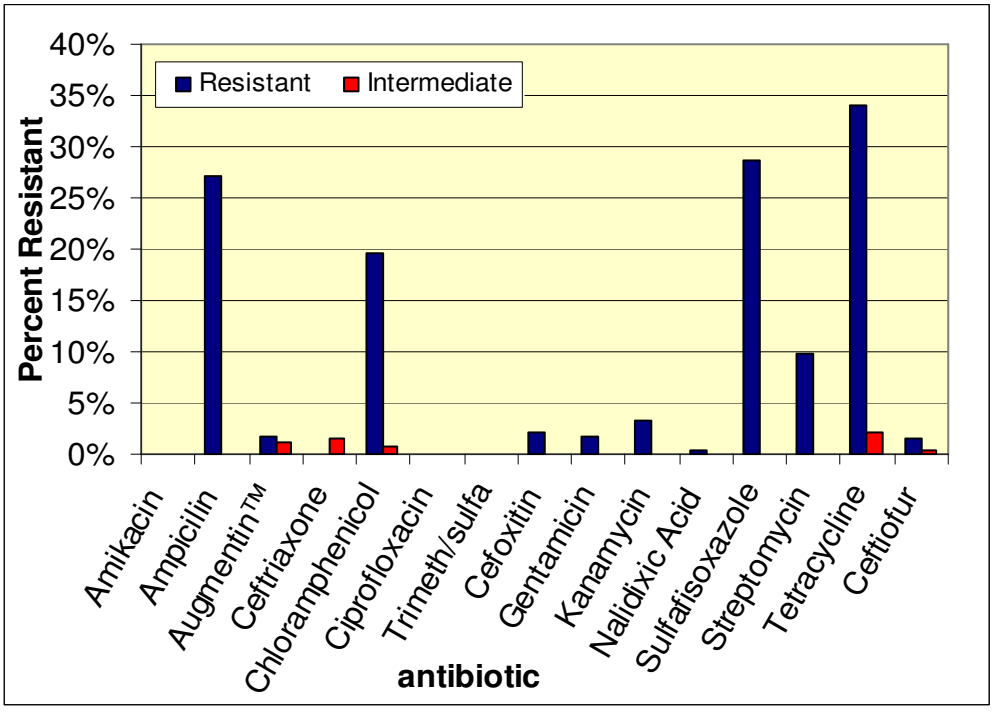
Overall, *Salmonella* isolates were resistant to fewer antibiotics than were *E. coli* isolates. The maximum number of antibiotics to which *Salmonella* isolates were resistant

was 6 (4 in water samples). In *E. coli* isolates, the maximum number of antibiotics to which there was resistance was 9 (8 in water samples).

Similar to resistance in *E. coli* isolates, there were no isolates that were resistant to all of the fifteen drugs studied. For the *Salmonella* isolates there were 4 drugs for which all isolates were susceptible. These included the two antibiotics for which the environmental *E. coli* isolates were susceptible: ciprofloxacin and amikacin, as well as ceftriaxone and trimethoprim/sulfamethoxazole.

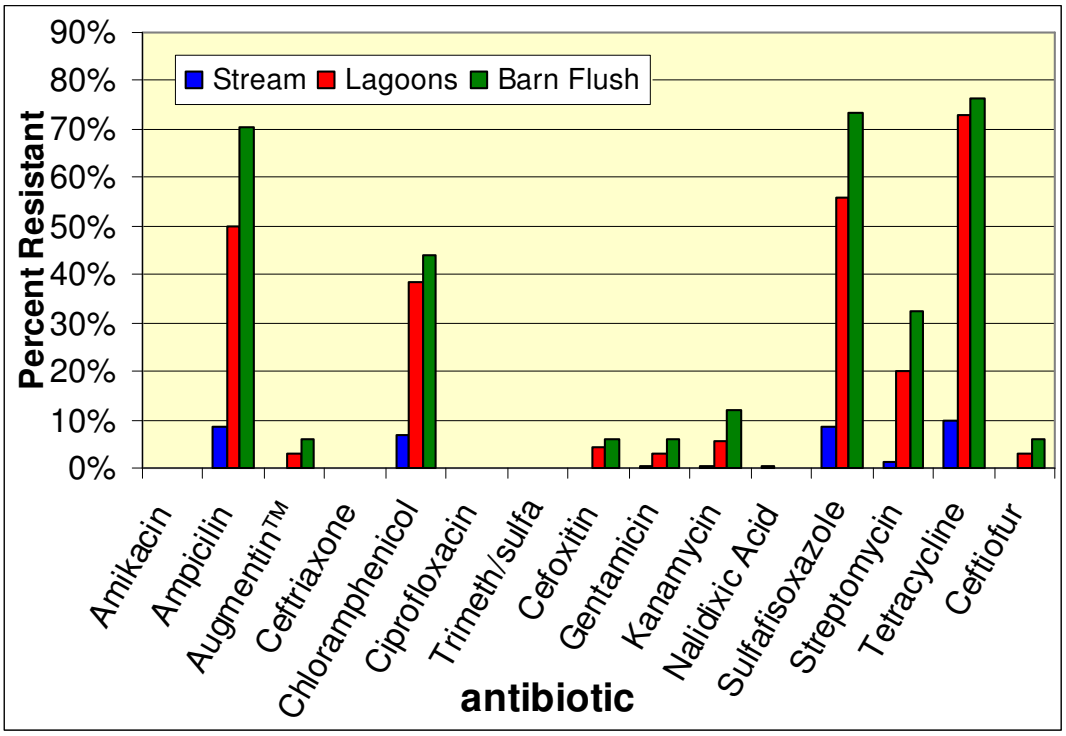
As with *E. coli,* tetracycline resistance was most frequent among *Salmonella* isolates (34%). Additionally, there were several other antibiotics for which the frequency of resistance in *Salmonella* isolates was at least 10%. These drugs were ampicillin, chloramphenicol, sulfisoxazole and streptomycin. (figures 5.7 and 5. 8).

Figure 5.7: Percent of Total Environmental *Salmonella* Isolates Resistant to the Various Antibiotics



The frequency of resistance to individual drugs was much higher among isolates from swine waste samples than from stream water samples (figure 5.8.). There were no instances for which greater than 10% of the *Salmonella* isolates collected from water were resistant to any one drug. Of the isolates collected from swine wastes, as much as 74% were resistant to an individual drug and there were 4 other drugs for which the frequency of resistance was greater than 20%.

Figure 5.8: Percent of Environmental *Salmonella* Isolates by Sample that are Resistant to Studied Antibiotics



Antibiotic Combinations in Multi-Drug Resistant Environmental Gram-Negative Isolates

Multi-drug resistance in Gram-negative isolates had definite patterns with regard to specific antibiotics and combinations of them. As previously noted, tetracycline resistance was most common and in the majority of isolates resistant to 3 or more drugs,

and it was accompanied by sulfisoxazole and ampicillin resistance. Resistance to these three drugs was seen in all *E. coli* isolates resistant to 6 or more antibiotics (12 isolates), 71% (15/21 isolates) of those resistant to 5 drugs, and 24% and 33% of those resistant to 3 and 4 antibiotics, respectively. All 44 *Salmonella* isolates resistant to 4 drugs were resistant to ampicillin, tetracycline, sulfisoxazole and chloramphenicol. All 15 isolates resistant to 5 antibiotics were resistant to tetracycline, sulfisoxazole, ampicillin, streptomycin and either chloramphenicol or kanamycin as the fifth drug. All but 2 of the 298 the Gram-negative isolates with resistance to 2 or more antibiotics had resistance to at least one of these three antibiotics.

Finding the combination of three drugs in multi-drug resistant strains of *E. coli* and *Salmonella* is consistent with previous findings by others. Resistance genes for these three antibiotics are often found together on plasmids (Oppegaard, H et al (2001), Herrero, A et al (2006), Hansen et al (2007), Shehabi A.A. et al (2006)). These plasmids are easily transferred among and between bacterial species in both environmental and hospital settings (Sunde M., and Sorum, H, (2001), Gebreyes, W.A. et al. (2006), Rijavec, M., et al. (2006) and Naiemi, NA et al, 2005)).

Environmental Enterococcus -

Antimicrobial analysis of *Enterococcus* species isolates is presented as two sub-topics relative to public health considerations: 1) resistance to antibiotics having human clinical significance (the drug marked "*" are used for treatment of enterococcal infections), including chloramphenicol, erythromycin, penicillin*, quinuprisitn/dalfopristin* (trade name Synercid™), tetracycline*, vancomycin*,

ciprofloxacin, linezolid*, gentamicin* and streptomycin*; and 2) resistance to antibiotics of significance primarily in veterinary medicine. The veterinary antibiotics include: tigecycline†, flavomycin, daptomycin†, lincomycin and tylosin tartrate ("†" these drugs have recently been approved for human use). Kanamycin and nitrofurantoin resistance was also analyzed but will be reported separately. This is because these antibiotics are used in human medicine but are not clinically relevant for enterococcal infections.

There were 418 *Enterococcus* sp. isolates collected from environmental samples. Of these, 174 isolates were from stream samples, 10 were from irrigation ponds, 5 from ground water wells, 101 from swine lagoon samples, 74 from swine barn flush samples and 54 from cattle manure.

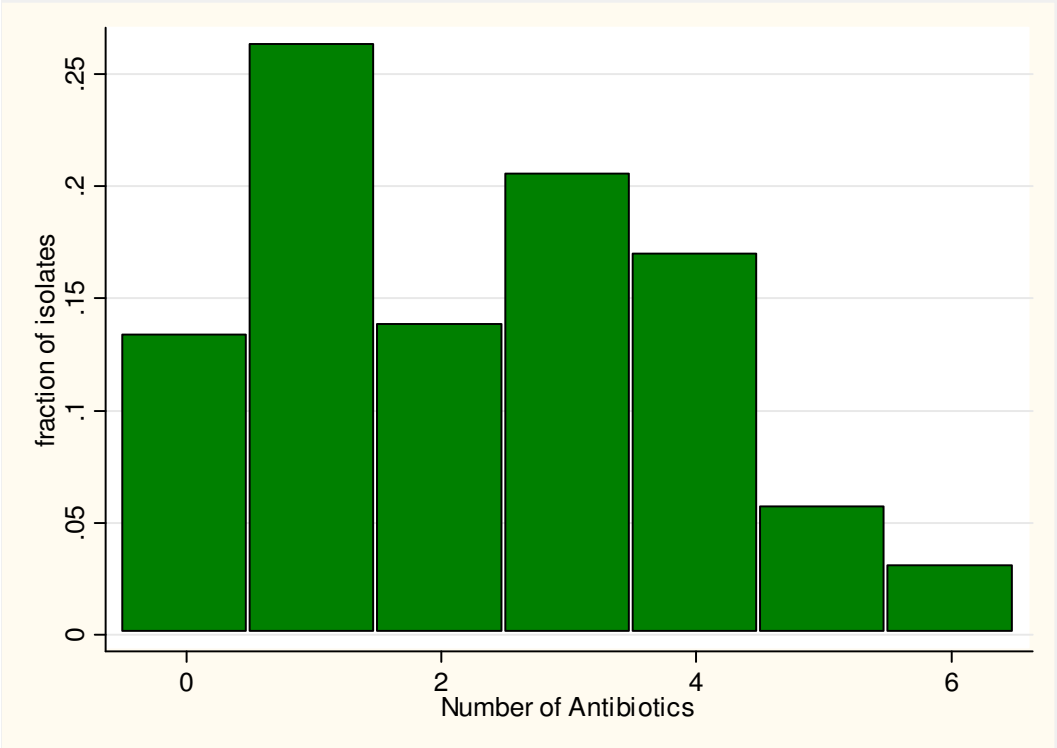
Antibiotic Resistance in Enterococcus to Human Clinically Significant Antibiotics

When examining resistance to drugs of clinical significance, there was a higher frequency of single and multiple antibiotic resistance in the environmental enterococci isolates than seen in *E. coli* and *Salmonella* environmental isolates. Of all the environmental *Enterococcus* sp. isolates, only 13.4% (56/418 isolates) lacked resistance to any of the 10 antibiotics of clinical significance, 26.2% (110 isolates) were resistant to only one of these antibiotics and about 60% were resistant to two or more of them (figure 5.9). Of the environmental *E. coli* and *Salmonella* isolates, 37% and 61%, respectively, were not resistant to any antibiotics, 28.5% and 7.6 %, respectively, were resistant to one antibiotic, and 34% and 32%, respectively, were resistant to two or more antibiotics.

Comparing the proportions of isolates resistant to 1 or more antibiotics, and proportion of isolates resistant to two or more antibiotics between the indicator species

(*E. coli* and *Enterococci*) it was found that there is a significantly higher proportion of *Enterococci* isolates that are mono- and multi-drug resistant than that in the environmental *E. coli* isolates (p values for both analyses are <0.0001).

Figure 5.9: Fraction of Total Environmental Enterococcus Isolates Resistant to Different Numbers of Clinically Important Antibiotics

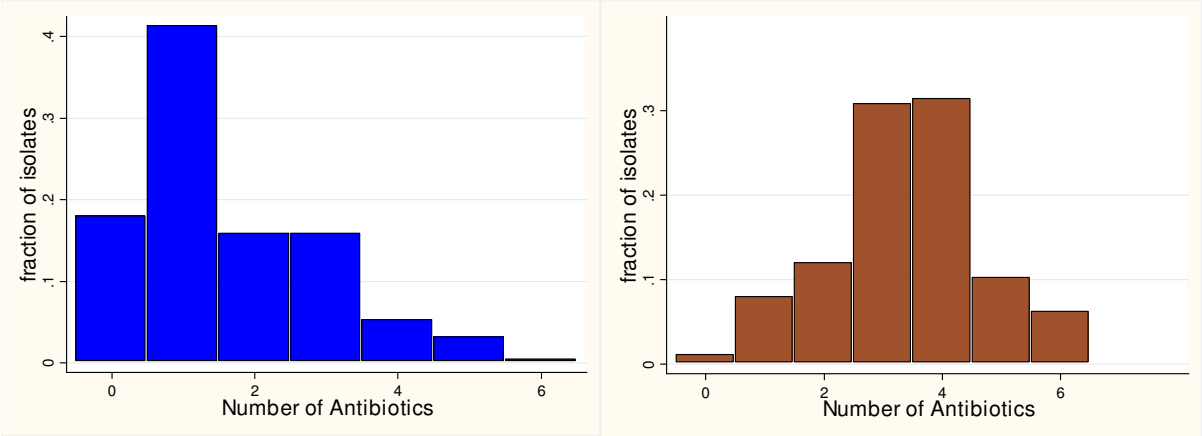


As with the Gram-negative bacteria, frequency of resistance in isolates by source was examined. Prevalence of *Enterococcus* sp. isolates with no resistance or resistance to only one antibiotic was higher in those isolates collected from stream water samples, 18% (32/174 isolates) and 43% (74/174 isolates), respectively, than those isolates collected from swine waste; 1% (2/175 isolates) resistant to no human clinical drug and 8% (14/175isolates) resistant to only one human clinical drug (figure 5.10a and b). These differences in single-and multi-drug resistance between isolates collected from waste

samples and those from water samples are statistically significant with p values of less than 0.0001. Overall, most of the *Enterococci* isolates in swine waste were resistant to 3 or 4 antibiotics, at frequencies of about 31% each; while the most of the *Enterococci* isolates from water were only resistant to one drug (43%).

Analysis of antibiotic resistance in *Enterococcus* isolates from the other water sources indicated that of the 10 isolates collected from irrigation ponds, two had no resistance, four were resistant to one drug, one was resistant to two drugs and three were resistant to three drugs. For the 5 Enterococcus isolates from ground water wells, four were resistant to three different antibiotics and one has resistant to four different antibiotics.

Figure 5.10: Fraction of Enterococcus Isolates obtained from Ground and Surface Water Samples (a), and from Swine Waste Samples (b) that are Resistant to Different Numbers of Antibiotics



a) Ground and Surface Water Samples **b) Swine Waste Samples**

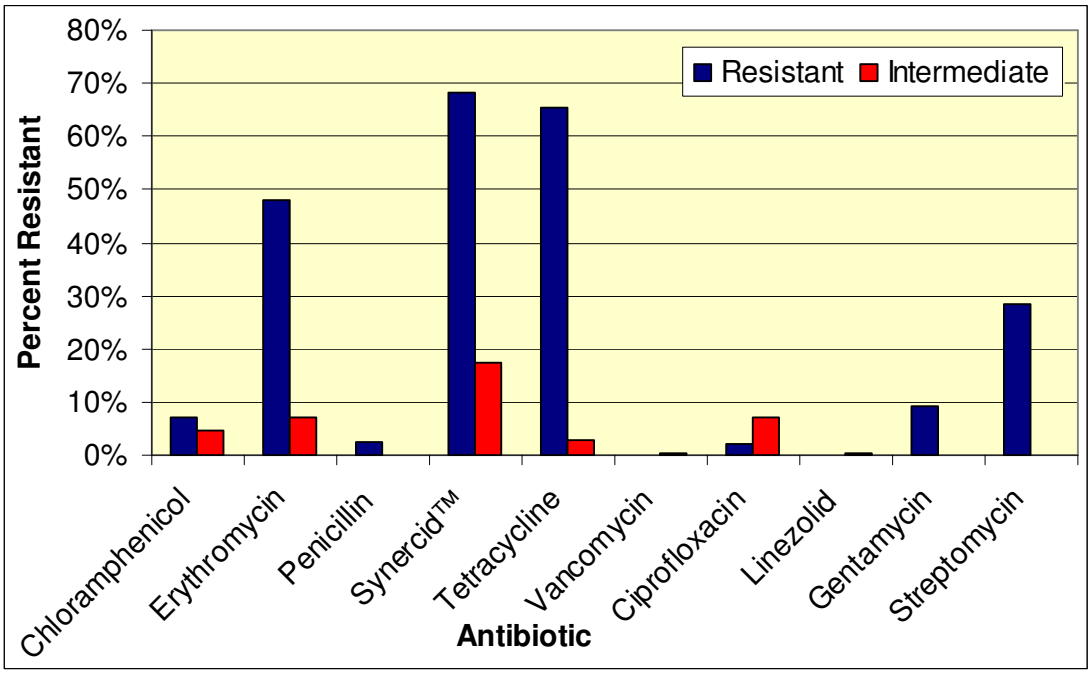
When examining all the environmental *Enterococci* isolates on the basis of which antibiotics they are most frequently resistant to, the two drugs with highest percentages of resistant isolates were quinuprisitn/dalfopristin with 68% of isolates resistant (plus

another 17% having intermediate resistance) and tetracycline, with 66% of the isolates resistant. (figure 5.11). Erythromycin and streptomycin also had high percentages of resistant isolates at 48% and 28%, respectively.

There were two clinically significant antibiotics for which no environmental Enterococcus isolates were resistant: linezolid and vancomycin. For both of these drugs, 2 isolates had intermediate levels of resistance, which represents less than 1% of the total isolates.

In contrast to the environmental Gram-negative bacteria isolates, some Enterococcus isolates had ciprofloxacin resistance, though the frequency of resistance was relatively low, at less than 5% of the total isolates. It is important to note that this is not a drug used in veterinary medicine but is clinically important therapeutically for treatment of some human bacterial infections (enterococcal infections are not treated with ciprofloxacin). Ciprofloxacin resistant enterococci were found in diverse samples, including stream water, swine waste and cattle manure.

Figure 5.11: Percent of Total Environmental Enterococcus sp Isolates with Resistance to Various Antibiotics of Human Clinical Significance

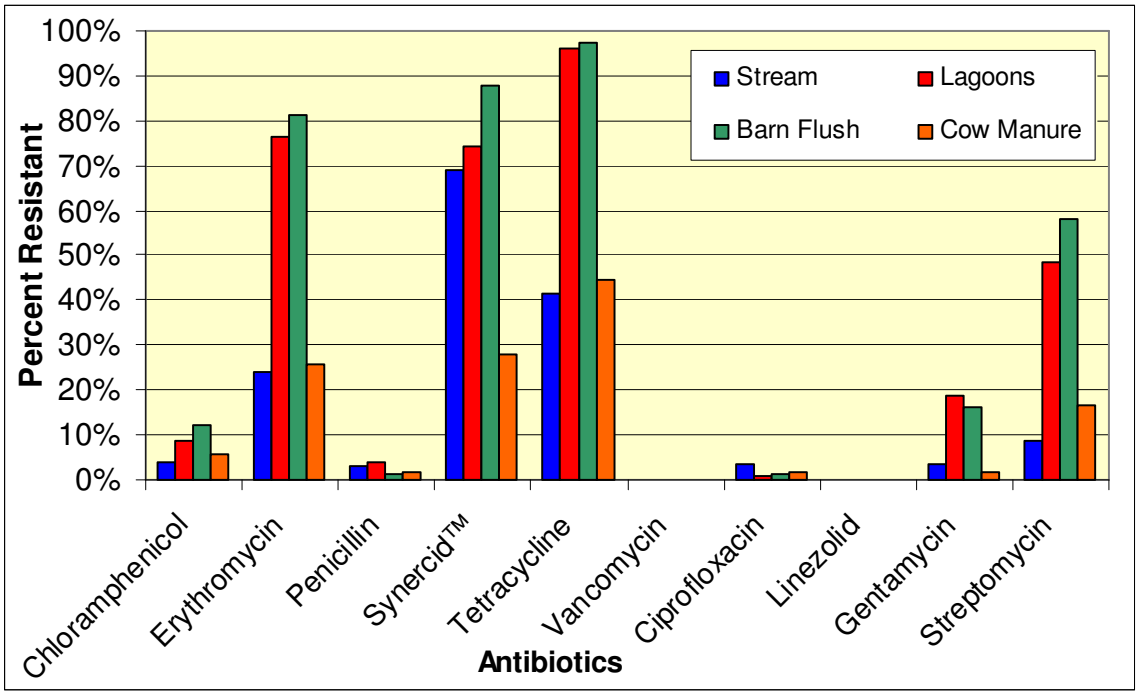


As in Gram-negative bacteria, Enterococcus resistance to individual drugs was higher for swine waste isolates than from cattle manure or stream water isolates. Nearly all of the barn flush (72/74 isolates) and lagoon isolates (97/101) were resistant to tetracycline; for erythromycin, 76% of lagoon sample isolates and 81% of barn flush isolates were resistant; and for quinuprisitn/dalfopristin, 74% of lagoon isolates and 88% of barn flush isolates were resistant. Additionally, greater than 10% of the isolates from barn flush and/or lagoon samples were resistant to chloramphenicol, streptomycin and gentamicin (figure 5.12).

Enterococcus isolates from stream water had different frequencies of antibiotic resistance than did the Gram-negative bacteria. Resistance in stream *Enterococcus* sp. isolates was much higher than that of the Gram-negative bacteria examined. For *Salmonella* sp. isolates, no drugs had greater than 10% frequency of resistance, and for *E.*

coli isolates, only tetracycline had greater than 10% frequency of resistance (19%). In contrast, for *Enterococcus* sp. stream isolates, almost 70% were resistant to quinuprisitn/dalfopristin, more than 40% were resistant to tetracycline and 28% were resistant to erythromycin.

Figure 5.12: Percent of Environmental Enterococcus Isolates Resistant to Various Antibiotics of Human Clinical Importance



<u>Enterococcus sp. Antibiotic Resistance of Veterinary Significance</u> –

Five antibiotics of this study were used solely in veterinary medicine: daptomycin*, flavomycin, lincomycin tigecycline* and tylosin tartrate (as previously mentioned daptomycin and tigecycline have recently been approved for human use however, the new breakpoints were established after the commencement of this study). Because these drugs were not used in human medicine, they did not have established resistance breakpoints. Instead, resistance is expressed as MIC_{50} and MIC_{90} values. An

MIC is the lowest concentration of an antibiotic that completely inhibits growth of the bacterium (NCCLS, 2001). Therefore, MIC_{50} and MIC_{90} values were determined based upon the concentrations of the antibiotics tested for which 50% and 90%, respectively, of the total *Enterococcus* sp. isolates (including those isolated from human and environmental sources) were inhibited. In some instances, the highest concentration of the drug analyzed did not inhibit the *Enterococci* isolates as high as 90%, and therefore "greater than (>)" values are reported. For example, the MIC_{90} value reported for flavomycin is >16 because at a concentration of 16μg/ml (the highest concentration analyzed) only 53% of the isolates were inhibited. Hence, 47% of the total *Enterococcus* sp. analyzed (isolated from human and environmental sources) were considered to be resistant to flavomycin at this concentration. In the case of lincomycin, only 22% of all *Enterococcus* sp. were inhibited at the highest concentration analyzed, i.e., 78% were "resistant" at the highest concentration of lincomycin analyzed. As a result, the MIC_{50} and MIC_{90} value are both reported at >32μg/ml.

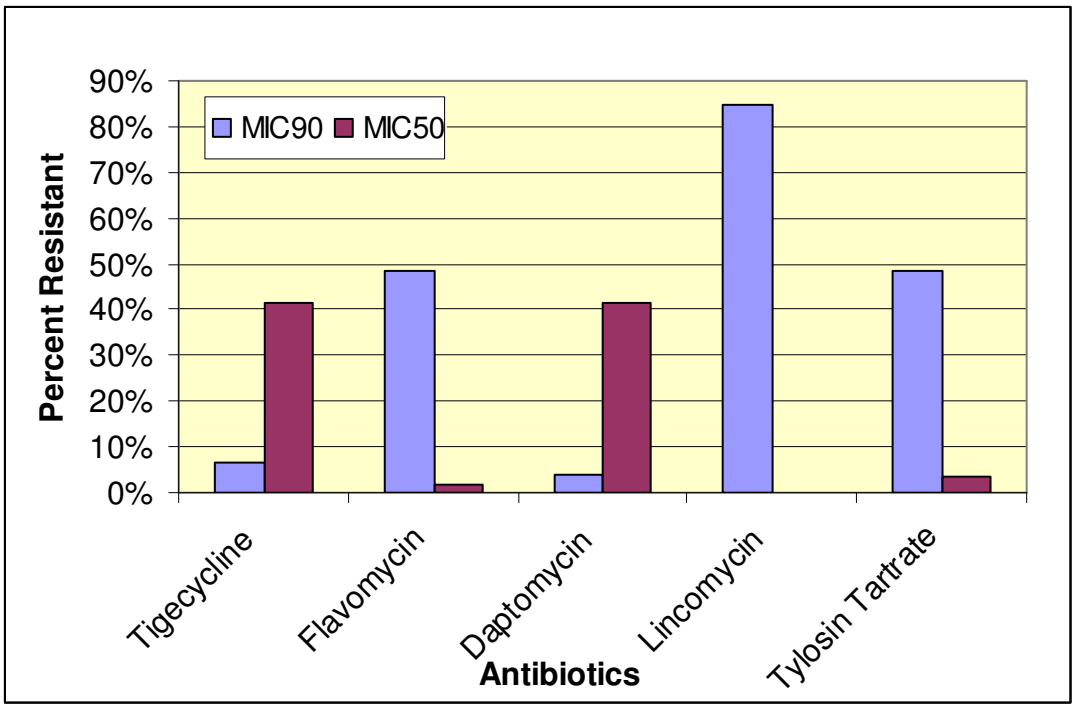
Table 5.4 lists the five different antibiotics, the MIC_{50} and MIC_{90} concentration values established, and the percent of the total *Enterococcus* sp., including isolates collected from human specimens that were inhibited at the given concentration. For purposes of further analysis, resistance will be considered at the MIC_{90} concentration, even when that value is a "greater than" value, and intermediate resistance corresponds to the MIC_{50} value.

Table 5.4: Percent of Enterococcus Inhibited at the concentration determined to be the MIC_{50} and MIC_{90} Values

Antibiotic	MIC_{50}(µg/ml) (% inhibited)	MIC_{90} (µg/ml) (% inhibited)
Daptomycin	1 (46%)	4 (96%)
Flavomycin	4 (50%)	>16 (53%)
Lincomycin	>32 (22%)	>32 (22%)
Tigecycline	0.25 (54%)	0.5 (95%)
Tylosin Tartate	4 (52%)	>32 (60%)

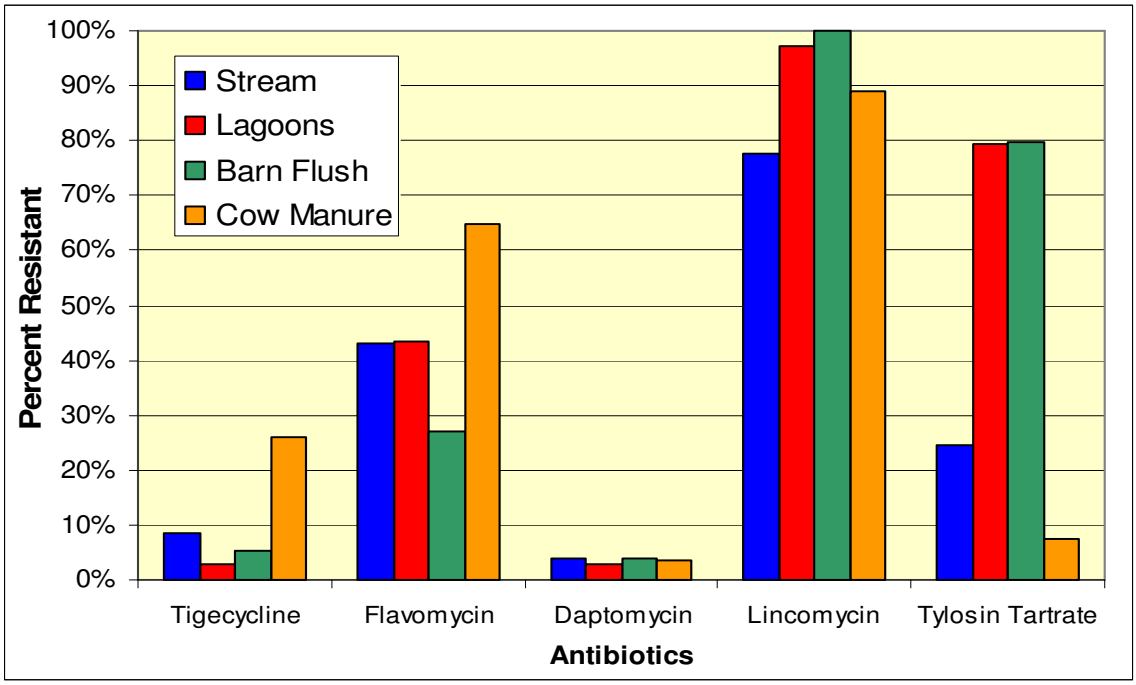
Using the breakpoint indicated in Table 5.4, 78%, 47% and 40% of the total *Enterococcus* sp. isolates are resistant to lincomycin, flavomycin and tylosin tartrate respectively. The percent of the total *Enterococcus* isolates resistant to tigecycline and/or daptomycin is much smaller, 5% and 4%, respectively. Of the *Enterococcus* isolated from environmental sources including animal waste and surface water samples, the frequency of resistance to these drugs is consistent with that of the overall Enterococcus sp. (figure 5.13). Almost 50% of the environmental Enterococcus sp. isolates are resistant to tylosin tartrate and flavomycin and more than 80% of the environmental Enterococcus sp. isolates are resistant to lincomycin.

Figure 5.13: Percent Environmental Enterococcus Isolates Resistant to Various Antibiotics of Veterinary Importance, as determined by MIC_{50} and MIC_{90} Values



Analyzing the Enterococcus isolates by sources (figure 5. 14), reveals that the frequency of resistance to daptomycin, tigecycline and flavomycin in environmental *Enterococcus* sp. was relatively consistent among the different sources (p= 0.9978, 0.1149 and 0.0629 respectively). However, Enterococcus sp. isolated from animal waste samples (including swine lagoons and barn flush samples, and cow manure samples) had higher frequencies of tylosin tartrate and lincomycin resistance than isolates from stream water samples ($p < 0.0001$ and $p=0.0003$, respectively).

Figure 5.14: Percent of Environmental Enterococcus Isolates Resistant to Antibiotics used for Veterinary Purposes by Sample Type



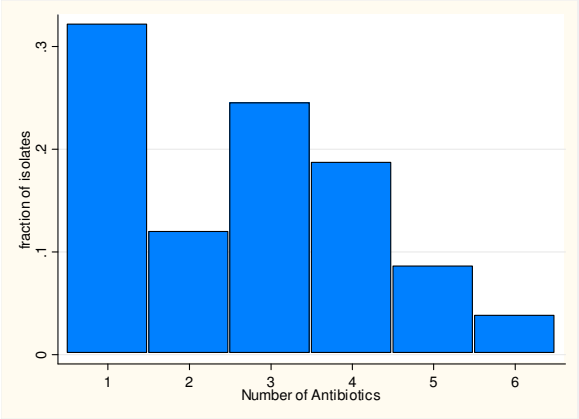
Analyses of Enterococcus sp. by the predominant species found: E. faecalis and E. faecium

The most common *Enterococcus* species in human specimens are *E. faecalis* and *E. faecium*, and therefore, it is important to understand antibiotic resistance frequencies in these species in environmental isolates. As stated in chapter 4, approximately 50% (235 isolates) of the environmental *Enterococcus* sp. are *E. faecalis*, and 14% (66 isolates) are *E. faecium*. Of these isolates, 208 *E. faecalis* and 60 *E. faecium* were analyzed for their antibiotic resistance profiles.

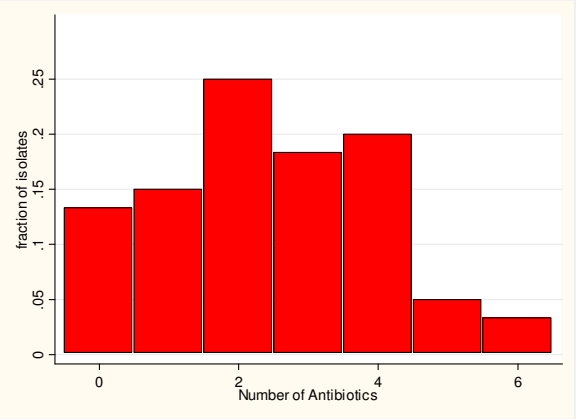
The overall frequency of isolates resistant to different numbers of antibiotics was generally the same in both species (figure 5.15a & b) (Kolmogrov-Smirnov test, p =0.379). However, one major difference is the number of isolates having no resistance.

Approximately 13% of the *E. faecium* isolates were susceptible to all clinically significant antibiotics (i.e. not resistant to any of these drugs). Among the *E. faecalis,* however, no isolates were susceptible to all drugs. Comparing these two species based upon proportion of isolates with resistance to at least one drug (i.e. resistance vs. no resistance), there is a statistically significant difference ($p < 0.0001$). There was no significant difference however, when comparing the proportion of multi-drug resistant isolates of *E. faecium* and *E. faecalis* ($p = 0.5683$). In other words, both *E. faecium* and *E. faecalis* had the same proportion of isolates with resistance to two or more drugs. The difference between species is in the number of isolates with no resistance at all. This indicates that the percentage of *E. faecalis* isolates resistant to one antibiotic approximates the percentage of *E. faecium* isolates resistant to one or fewer antibiotics.

Figure 5.15a and b: Fraction of *E. faecalis* (a) and *E. faecium* (b) Resistant to Different Numbers of Clinically Significant Antibiotics



a) *E. faecalis*

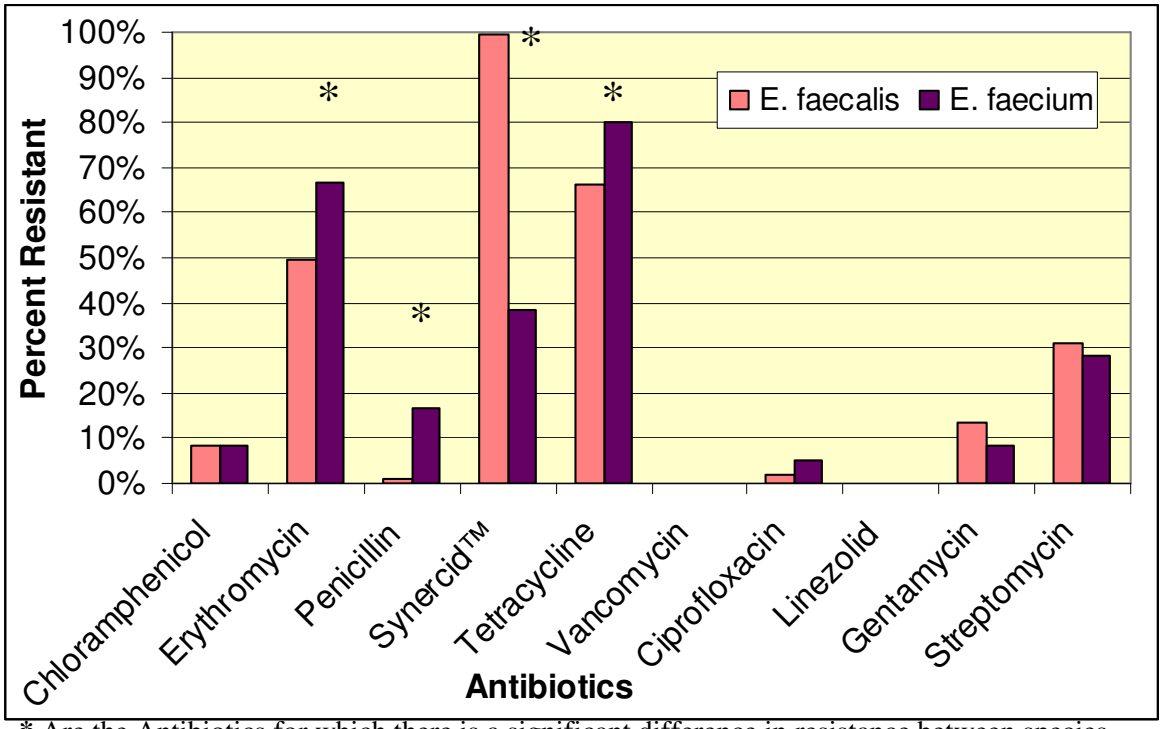


b) *E. faecium*

While overall frequency of the multi-drug resistance was not significantly different between the two species, analyses reveal that there are differences in the frequency of resistance to specific drugs by species. One hundred percent of the *E.*

faecalis isolates but only 40% of the *E. faecium* were found to be resistant to quinupristin/dalfopristin; the difference in proportions is statistically significant p<0.0001) (figure 5. 16). There was penicillin resistance in nearly 20% *E. faecium* isolates but only about 1% in *E. faecalis* isolates (p=0.0003). There was only a small, but significant difference between these species in the frequency of tetracycline and erythromycin resistance (p =0.0432 and p = 0.0190), with 66% and 50% resistant, respectively, in *E. faecalis* and 80% and 67%, resistant respectively, in *E. faecium*. The frequency of resistance to streptomycin, gentamicin, ciprofloxacin and chloramphenicol was similar between the two species.

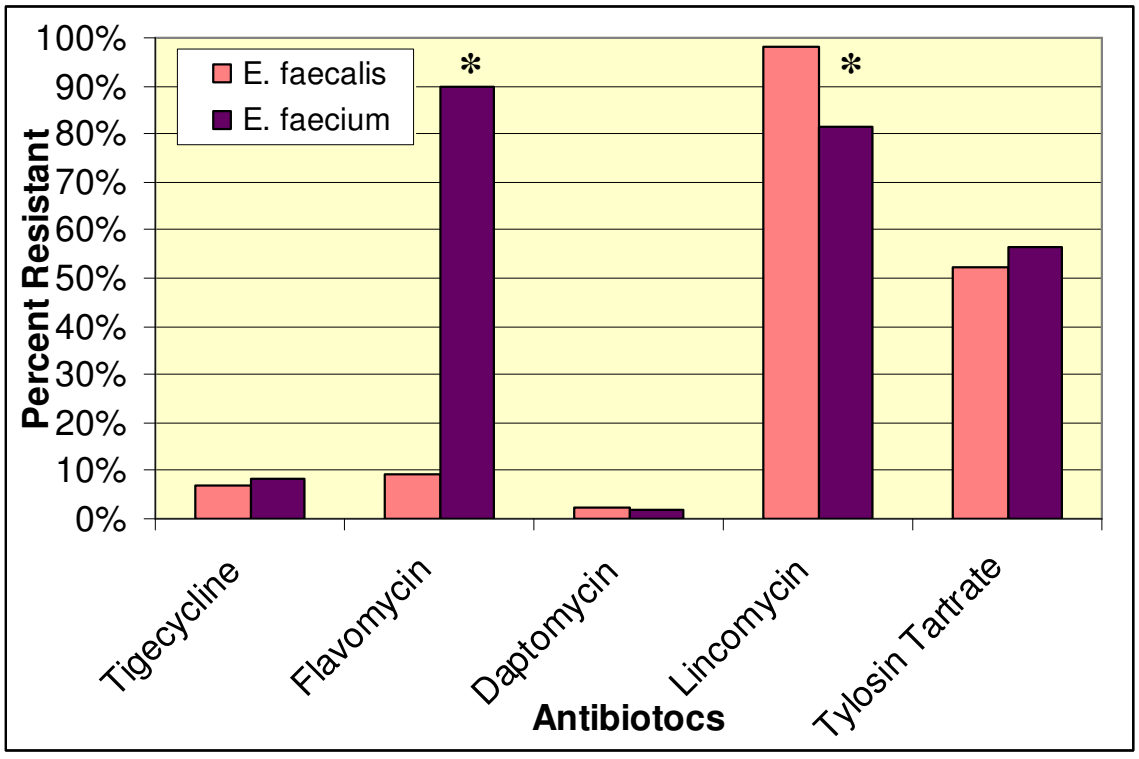
Figure 5.16: Percent of *E. faecalis* and *E. faecium* Isolates Resistant to Various Human Clinically Significant Antibiotics



* Are the Antibiotics for which there is a significant difference in resistance between species

In addition to the differences in resistance in the two most common *Enterococcus* species to clinically significant human drugs, resistance in *E. faecalis* and *E. faecium* to drugs of veterinary significance were compared. Resistance frequencies in tigecycline, daptomycin and tylosin tartrate were about the same in both species (p = 0.6700, 0.7338 and 0.5598, respectively), however, the proportions of isolates resistant to flavomycin and lincomycin were significantly different in the two species. Almost 90% of *E. faecium* isolates were resistant to flavomycin compared to less than 10% resistance in *E. faecalis* isolates to this drug. Nearly 100% of the *E. faecalis* isolates were resistant to lincomycin, while only about 80% of the *E. faecium* were resistant to the drug (figure 5. 17).

Figure 5.17: Percent Environmental *E. faecalis* and *E. faecium* Isolates Resistant to Various Antibiotics of Veterinary Significance



* Are the Antibiotics for which there is a significant difference in resistance between species

Antibiotic Combinations in Multi-Drug Resistant Environmental Enterococcus Isolates

Resistance to multiple antibiotics was common among the isolates (see Appendix A for a table of profiles). Overall, resistance to quinupristin/dalfopristin and tetracycline was the most frequent. All but one isolate resistant to two clinically significant antibiotics were resistant to tetracycline (53/58 (91%) isolates), quinuprisitn/dalfopristin (27/58 or 47%) or both. Erythromycin resistance was also very common among the multi-drug resistant isolates, with 23 of 58 (40%) of these isolates resistant to erythromycin.

Of isolates resistant to three clinically significant antibiotics, 66% were resistant to quinuprisitn/dalfopristin, tetracycline and erythromycin, and of those resistant to 4 or more clinically significant drugs more than 95% were resistant to these three drugs.

Resistance to high level aminoglycosides such as streptomycin and gentamicin was common among those Enterococcus isolates with resistance to four or more drugs. All isolates resistant to six clinically significant drugs had resistance to high levels of streptomycin and all but one was also resistant to high levels of gentamicin. All isolates resistant to five clinically significant drugs were resistant to streptomycin, gentamicin or both. Seventy-nine percent of isolates resistant to four antibiotics were resistant to high levels of streptomycin, and in addition to these 79% with streptomycin resistance, 4% of the isolates were resistant to high levels of gentamicin.

When considering antibiotics significant in both veterinary and human medicine, almost all isolates resistant to 4 or more clinically significant drugs also were resistant (as established by MIC_{90} values) to flavomycin, lincomycin and tylosin tartrate. A much lower number of isolates were also resistant to daptomycin and tigecycline. No isolates

were resistant to at least one human clinically significant antibiotic and all five of the veterinary antibiotics. However, eight isolates were resistant to 2 or more human clinically significant antibiotics and four of the five veterinary drugs, with five of these resistant to flavomycin, lincomycin, tylosin tartrate and daptomycin, and the remaining three resistant to flavomycin, lincomycin, tylosin tartrate and tigecycline.

As seen in antibiotic resistance in the Gram-negative bacteria, there are predominant patterns of resistance in the multi-drug resistant *Enterococcus* sp. Most of the multi-drug resistant *Enterococcus* sp. are resistant to quinupristin/dalfopristin, erythromycin, lincomycin, tylosin tartrate and/or tetracycline. There are several different mechanisms of resistance to these drugs. Some of the resistance is intrinsic by species, as previously discussed. However, there is one mechanism that mediates resistance to all of the first four drugs. Without molecular characterization of the isolates to determine which resistance genes are present, the mechanism(s) of resistance cannot be determined. Without this further characterization conclusive links to specific origins of the bacteria cannot be made.

Comparison of Antimicrobial Resistance in Stream Water Enteric Bacteria by Farm Type

To fully assess the impact of antibiotic resistance originating from animal agriculture facilities on environmental waters, it is essential to examine the frequency of single and multi-drug resistance of enteric bacteria by stream sample type. Four types of stream water samples were compared: upstream of row crop farms, downstream of row crop farms, upstream of animal agriculture, and downstream of animal agriculture. To

determine the impact of farm type, antibiotic resistance in bacteria upstream from animal agriculture facilities are compared with those downstream of animal agriculture facilities, and bacteria upstream of row crop farms were compared with those downstream of row crop farms. The differences in the distributions of the number of antibiotics to which isolates from each water sample type are taken were compared. Then, to compare the differences in bacterial antibiotic resistance between the two farm types, bacteria in downstream samples from animal agriculture facilities were compared with bacteria in downstream samples of row crop farms.

For the first two comparisons, upstream and downstream, a statistic for dependent variables was used. This is because the upstream and downstream samples at each farm may be correlated, because they are taken from the same stream. The Wilcoxon Signed Rank test assesses the difference in the medians of two dependent populations; this is a paired analysis. Unfortunately, the isolates collected from the stream water are not paired. While every effort was made to collect the same number of isolates from each sampling site, there were instances in which one type of bacterium was not present in a given sample or, it was not possible to isolate that type of bacterium from the sample. As a result, while bacterial concentration data described in chapter 4 can be subject to pairwise analyses, not all of the bacterial isolate data can be subject to pairwise analyses. Furthermore, while it is acknowledged that up- and down- stream samples around each farm are from the same streams and therefore potentially correlated, it is possible that the data are in fact independent. If each bacterial isolate is treated as an individual, and all of the bacteria from the stream are the potential source population, the probability that a clone of a bacterium collected upstream is also collected several hundred meters

downstream may be low. Therefore, a proportion test, which analyzes the difference in the means of independent binomial populations, was used as well as the Wilcoxon Signed Rank test. To apply the proportion test, isolates of each species of bacteria were divided into groups. For an initial comparison, the isolates are scored zero if they had no resistance, and one if there was resistance to one or more bacteria. In addition, another binomial variable was established for multiple antibiotic resistance, with an isolate scored zero for no resistance or resistance to only one drug, and scored one if the isolate had resistance to two or more antibiotics. In the case of the Enterococcus isolates, only multiple resistance is compared as many of the Enterococcus species have intrinsic resistance to at least one antibiotic and therefore, almost all of the isolates are resistant to at least one drug.

For comparison between animal agriculture water samples and row crop water samples a test that assesses independent populations may be used. This is because the streams from which samples were collected were generally different streams or far from one another. As with the Wilcoxon Signed Rank test, the Wilcoxon Rank Sum test (also known as the Mann-Whitney test) assesses the difference in the frequency distribution of isolates resistant to different numbers of antibiotics, ranging from zero to nine antibiotics based upon the median value in each of the two groups. This is not a paired comparison and is used for independent populations. In this analysis, the distribution of isolates collected downstream of row crop farms was compared to that of the isolates collected downstream of CAFOs

An additional test that was used to compare populations is the two sample Kolmogrov-Smirnov test for equality of distribution functions . This statistical test

examines the entire distribution of each population of isolates to determine any differences. The advantage of this test versus the Wilcoxon Rank Sum test is that it considers the entire distribution rather than the median alone. Therefore, distributions that may have the same medians but different skews or dispersions may be more accurately compared. There is no comparable test to the Kolmogrov-Smirnov test for dependent populations. Lastly, the proportion test for binomial outcomes was also used to compares the means of the data. As with the comparisons among farm type, the variables are coded as two binomial variables, one for resistance to one drug and the other for multi drug resistance.

All four stream types were analyzed (aggregate results discussed earlier in the chapter) by bacterial species. The percentage of bacterial isolates by species from each stream sample type with single, multiple and no resistance was determined (Table 5.5a , b & c and figures 5.18 a, b, c and d).

Table 5.5 a,b,c: Percent of Stream Samples Bacteria Isolates having Single and Multi- Drug Resistance

a. *E. coli*

Sample Type	No Resistance		Resistant to ONE or more Drugs		Resistant to TWO or more Drugs	
	# of isolates	percent	# of isolates	percent	# of isolates	percent
Row Crop Up	31	74%	11	26%	1	2.4%
Row Crop Down	30	75%	10	25%	3	7.5%
Animal Ag Up	25	51%	24	49%	7	14%
Animal Ag Down	42	62%	26	38%	12	18%

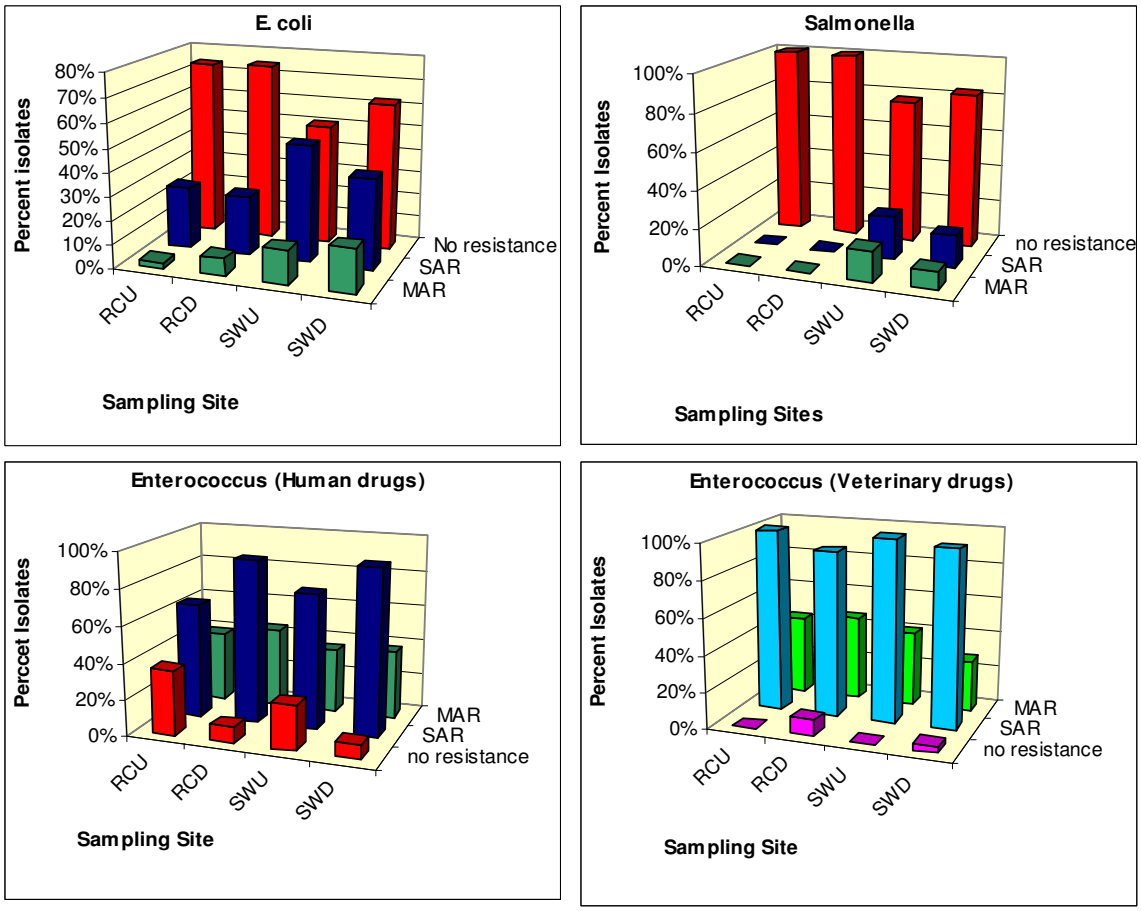
b: *Salmonella*

Sample Type	No Resistance		Resistant to ONE or more Drugs		Resistant to TWO or more Drugs	
	n	percent	N	percent	n	percent
Row Crop Up	29	100%	0	0%	0	0%
Row Crop Down	32	100%	0	0%	0	0%
Animal Ag Up	34	77%	10	23%	7	16%
Animal Ag Down	50	83%	10	17%	6	10%

c: *Enterococcus*

Sample Type	No Resistance				Resistant to ONE or more Drugs				Resistant to TWO or more Drugs			
	Clinical		***Vet***		***Clinical***		***Vet***		***Clinical***		***Vet***	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Row Crop Up	13	(36)	0	(0)	23	(64)	36	(100)	14	(39)	16	(44)
Row Crop Down	3	(9)	3	(9)	31	(91)	31	(91)	15	(44)	16	(47)
Animal Ag Up	11	(25)	0	(0)	33	(75)	44	(100)	16	(36)	18	(41)
Animal Ag Down	5	(8)	2	(3)	55	(92)	58	(97)	23	(38)	23	(28)

Figures 5.18 a, b, c* &d*: Percent of Stream Samples Bacteria Isolates having Single and Multi- Drug Resistance



*z axis (resistance proportion) reversed for the Enterococcus graphs to improve visibility

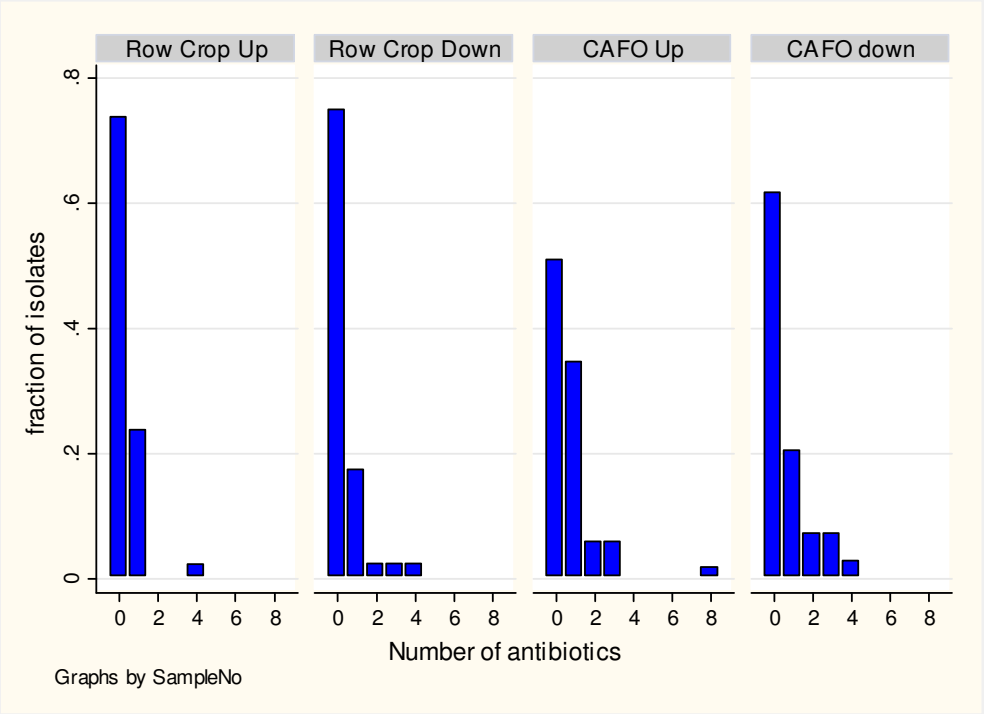
To assess the contribution of antibiotic resistant bacteria for each farm type, the frequency distributions of isolates in downstream samples for each bacterial species/genus were compared with the upstream sample for each type of farm with statistical analyses as described above. Next, to assess the impact of animal agriculture on the resistant bacteria in stream water as compared with the impact of non animal agriculture farms on resistant bacteria in stream water, the frequency of resistant bacteria downstream of animal agriculture facilities was compared to that downstream of the row crop facilities.

The majority of the Gram-negative bacteria in all stream types (row crop farm upstream and downstream, and CAFO upstream and downstream) are not resistant to any antibiotics. This includes 64% of *E. coli* isolated from stream water (128/199 isolates) and 88% of *Salmonella* isolates (145/165 isolates). In contrast, no antibiotic resistance among the *Enterococcus* sp. was infrequent. Only 18% of the *Enterococcus* sp. isolates (32/174) had no resistance to any of the human clinically significant drugs; and when examining single and multi-drug resistance in *Enterococcus* isolates, combining the clinically significant antibiotics and the veterinary drugs, only 1 isolate of the 174 (0.6%) collected from stream water was NOT resistant to any of the 15 different antibiotics. More than 75% of the *Enterococci* isolates from each of the four stream sampling types were resistant to 2 or more antibiotics of the combined two types of drugs.

Comparing Antibiotic Resistance of E. coli among Stream Samples

When the number of antibiotic resistance traits in *E. coli* isolates was examined by stream sample type, the majority (64%) of isolates are not resistant to any antibiotic (figure 5.19). Of the remaining, resistant *E. coli* isolates, most (48 isolates) are resistant to only one antibiotic. Only a small fraction is resistant to multiple antibiotics (11.5%), with isolates resistant to 2 up to 8 drugs.

Figure 5.19: Frequency Distribution of *E. coli* Isolate Resistance to Different Numbers of Antibiotics by Stream Water Samples



When *E. coli* isolates collected upstream of row crop farms are compared with those downstream, there is no statistical difference with regard to antibiotic resistance frequency (Wilcoxon Signed Rank test; p value = 0.7436) (Table 5.6). However, when assessing the efficacy of matching for the Wilcoxon Signed Rank test, the Spearman correlation coefficient was negative(r = -0.1015), indicating that a paired analysis is not effective. Because the paired analysis was not appropriate, the result may be questionable. When analyzing the same upstream and downstream antibiotic resistance frequency data using the unpaired Wilcoxon Rank Sum test, the difference was still not statistically significant (p = 0.8546). Using the binomial proportion test, to analyze for difference with regard to any resistance or differences in multiple resistance, no

significant differences were seen in the two populations (p = 0.9017 mono- resistance and 0.2821 multiple resistance).

When comparing frequencies if multi-drug resistant *E. coli* in upstream and downstream samples of CAFOs by binomial proportion test the two populations also were not different with respect to resistance to one or more drugs or resistance to two or more drugs (p = 0.2464 and 0.6267, respectively). There was a significant difference in frequency of antibiotic resistance of isolates upstream and downstream by the Wilcoxon Signed Rank test (p =0.0004). However, the Spearman correlation coefficient was negative (r = -0.1052) and therefore the pairing in this analysis was not effective. Using a non-paired Wilcoxon Rank Sum test, the difference in the two populations was not significant (p = 0.4663).

Comparing antibiotic resistance frequencies of bacteria in downstream samples by farm type, there is no significant difference in their resistance frequencies by any of the statistical comparisons used (p > 0.05) (Table 5.6). This includes examining the differences in median values using the Wilcoxon Ranked Sum test; comparing means of the two populations with regard to any resistance or multiple resistance; and comparing the total distribution of populations with regard to the number of antibiotics to which the isolates are resistant using the Kolmogrov-Smirnov test.

Table 5.6: Results of Various Statistical Tests (as P-values) Comparing the Frequency of Antibiotic Resistance among *E. coli* Isolates from Various Stream Sample Sites

	Sampling Site Comparisons - (p value)		
Test	**Downstream sites**	**Row Crop sites**	**Animal Ag Sites**
Wilcoxon Signed Rank (pairing effective Y/N)	--	0.7436 (No)	**0.004 (No)**
Wilcoxon Rank Sum	0.2042	0.8546	0.4663
Kolmogrov-Smirnov	0.699	1.0	0.850
Binomial Proportion (SAR*)	0.1588	0.9017	0.2464
Binomial Proportion (MAR†)	0.1409	0.2821	0.6267

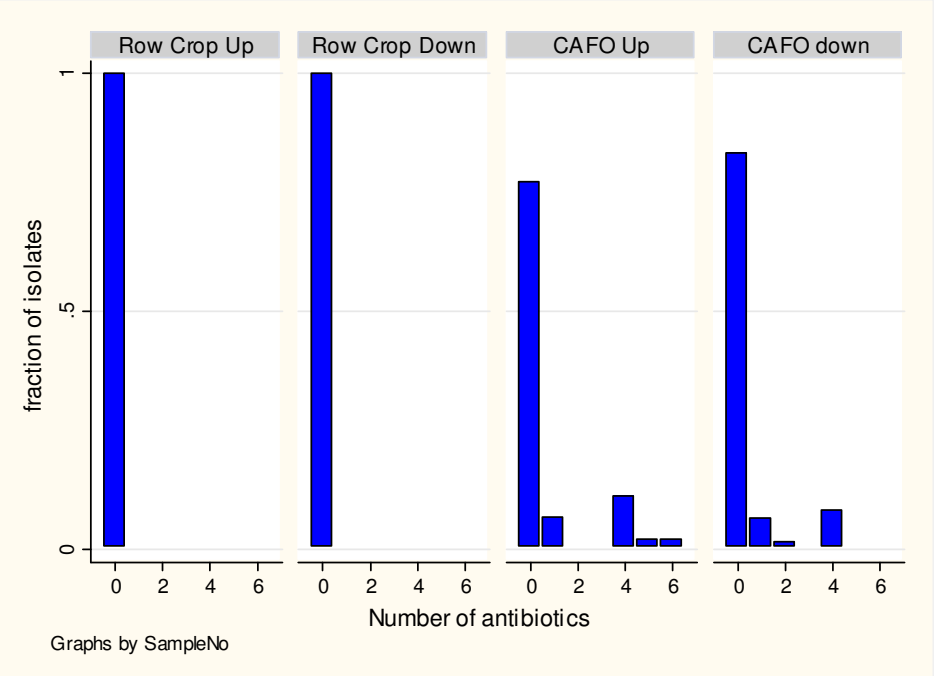
*SAR compares proportions of no resistance to resistance to one or more drugs

† MAR compares proportion of resistance to zero or one drug to those with resistance to 2 or more drugs

Comparing Antibiotic Resistance of Salmonella among Stream Samples

When *Salmonella* isolates from various stream samples sites were compared for the frequencies of antibiotic resistance, the majority of isolates (88%) lacked resistance to any antibiotics (figure 5.20), as was found for *E. coli* isolates. *Salmonella* isolates collected from stream samples of row crop farms were not significantly different in their distributions of antibiotic resistance. Indeed, there were no isolates with resistance in either upstream or downstream samples of row crop farms. For this reason, some statistical comparisons done for *E. coli* isolates were not possible with *Salmonella* isolates, as standard deviations were zero. The Wilcoxon Ranked Sum test to compare the downstream samples by farm type and the tests to compare differences in Salmonella resistance frequencies in upstream and downstream samples of row crop farms were not possible, as there were clearly no differences.

Figure 5.20: Frequency Distribution of *Salmonella* Isolate Resistance to Different Numbers of Antibiotics by Stream Water Sample



Of the Salmonella isolates associated with animal agriculture, there was no statistically significant difference in antibiotic resistance frequency, based on comparing the medians of upstream and downstream samples (p value = 0.5116). There were also no significant differences between upstream and downstream sites when comparing Salmonella isolate antibiotic resistance frequencies between based on the total distribution using the Kolmogrov-Smirnov test (p value = 0.994) or examining proportions of isolates with any resistance (p value = 0.4385) or multiple resistance (p value = 0.3680) (table 5.7).

When comparing Salmonella isolate resistance to one or more antibiotics in downstream samples by farm type, there is a statistically significant difference in the

frequency of resistance between swine animal agriculture facilities and row crop farms (p value = 0.0144). However, there was no significant difference when comparing the proportion of downstream *Salmonella* isolates with multiple drug resistance by farm type, (although the p value of 0.0643 approached the alpha = 0.05 level) or when comparing the entire distribution by farm type (p = 0.530). As mentioned above, it was not possible to compare antibiotic resistances frequencies in *Salmonella* isolates collected downstream from row crop farms to those collected downstream of CAFOs based upon medians with the Wilcoxon test Ranked Sum test, as the standard deviations within the downstream row crop sampling site was zero.

Table 5.7: Results of Various Statistical Tests (as p values) Comparing the Frequency of Antibiotic Resistance among *Salmonella* Isolates from Various Stream Samples

	Sampling Site (p value)		
Test	**Downstream sites**	**Row Crop sites**	**Animal Ag Sites**
Wilcoxon Signed Rank (pairing effective Y/N)	--	n/a	n/a
Wilcoxon Rank Sum	n/a	n/a	0.5116
Kolmogrov-Smirnov	0.530	1.0	0.994
Binomial Proportion (SAR*)	**0.0144**	n/a	0.4385
Binomial Proportion (MAR†)	0.0643	n/a	0.3680

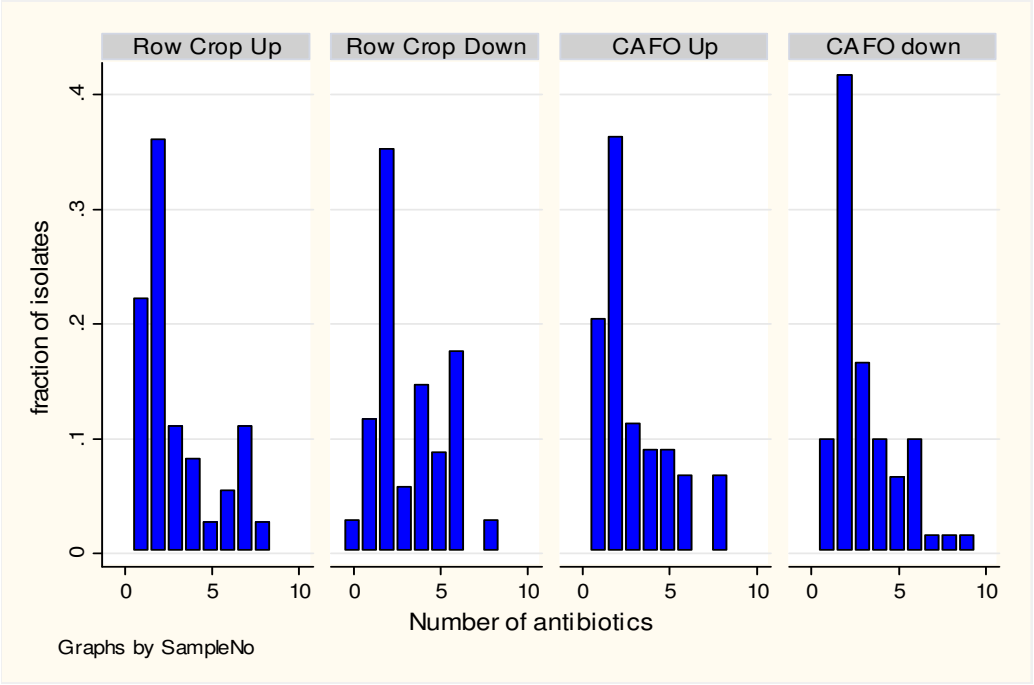
*SAR compares proportions of no resistance to resistance to one or more drugs
† MAR compares proportion of resistance to zero or one drug to those with resistance to 2 or more drugs

Comparing Antibiotic Resistance of Enterococcus among Stream Samples

Unlike the Gram-negative bacteria examined, different *Enterococcus* species have some intrinsic antibiotic resistance. As a result, almost all enterococcal isolates (99%)

collected from stream water had resistance to one or more antibiotics of human clinical or veterinary significance (figure 5.21). Therefore, when making the comparisons of frequencies of antibiotic resistance in *Enterococcu*s isolates using the binomial proportion tests only the multi-drug resistant analyses was conducted. All other data analyses are similar to those conducted for the Gram-negative bacteria.

Figure 5.21: Frequency Distribution of *Enterococcus* Isolate Resistant to Different Numbers of Human Clinically Significant and Veterinary Antibiotics, by Stream Water Sample



The *Enterococcus* antibiotic resistance distributions of the upstream and downstream samples of row crop farms was the only comparison for which pair-wise analyses was effective. When performing the Wilcoxon Signed Rank test (for matched pairs) the Spearman correlation coefficient (r) was 0.3983, and the p value to determine if the matching was effective was 0.0108, indicating the matching was effective. Therefore,

the Wilcoxon Signed Rank test was appropriate to use and the difference in the two populations was found to be not statistically significant (p value = 0.2600). To be consistent with the other comparisons for *E. coli* and *Salmonella*, however, the other statistical tests were performed. In all of the statistical analyses performed, there were no statistically significant differences found between the antibiotic resistance frequency distributions of *Enterococcus* isolates collected upstream of row crop farms compared with those isolates collected down stream of row crop farms (Wilcoxon Rank Sum test, p value = 0.3687, Kolmogrov-Smirnov p value = 0.859, and the binomial proportion test p value = 0.4190).

Pair-wise analyses of *Enterococcus* antibiotic resistance among the animal agriculture stream sampling sites were found not to be effective. The Spearman correlation coefficient was 0.1257 and the p value for the efficacy of pairing was 0.2110. However, if the pair-wise analysis is conducted despite pairing efficacy, the difference in antibiotic resistance frequencies of *Enterococcus* isolates between the up and downstream samples of CAFOs is not considered significant (p value = 0.8784). Using statistical tests for independent samples, there were also no statistically significant differences in the prevalence or distribution of antibiotic resistance among *Enterococcus* isolates from different stream samples (table 5.8 Animal Ag Sites).

Comparing the *Enterococcus* isolates collected downstream of CAFOs to those collected downstream of row crop farms there were also no significant differences detected by any of the statistical analyses employed (table 5.8 downstream sites).

Table 5.8: P Results of Various Statistical Tests (as p values) Comparing the Frequency of Antibiotic Resistance among *Enterococcus* Isolates from Various Stream Samples

	Sampling Site Comparison - p value		
Test	Downstream sites	Row Crop sites	Animal Ag Sites
Wilcoxon Signed Rank (pairing effective Y/N)	--	0.2600 (Yes)	0.8785 (No)
Wilcoxon Rank Sum	0.7404	0.3687	0.3926
Kolmogrov-Smirnov	0.841	0.859	0.916
Binomial Proportion (MAR†)	0.4952	0.4190	0.1338

† MAR compares proportion of resistance to zero or one drug to those with resistance to 2 or more drugs

Summary of Antibiotic Resistance Comparisons among Enteric Bacteria in Different Stream Water Samples

Comparing the occurrence and distribution of antibiotic resistance in the three different bacterial genera by stream water sampling site revealed no statistically significant differences when comparing bacteria isolated from upstream and downstream samples with in farm types. Frequency histograms showed there to be some differences in overall occurrence of resistance to one or more antibiotics in each of the sampling sites, however, these differences were not statistically significant. This conclusion is based on results of four different statistical tests, considering both dependence and independence of the sampling sites, as well as statistical comparisons based upon median values of antibiotic resistance frequency, the entire distributions of resistance frequency and comparisons of resistant proportions in each population as mean values. This evidence suggests that there is little or no difference in the impact of different farm types on the presence of antibiotic resistant bacteria in stream waters. That is, there is little or no statistical evidence that the extent of antibiotic resistant bacteria present in stream

waters is clearly influenced by entrance or impacts from specific row crop farms or swine animal agriculture facilities.

When comparing frequencies in the extent of resistance of bacterial isolates in downstream waters of animal agriculture facilities versus row crop farms, there were some numerical differences, however, in most cases these differences were not found to be statistically significant. The only case in which a significant difference was seen was in the comparison of *Salmonella* isolate resistance to one or more antibiotics by farm type. There was a higher proportion of *Salmonella* isolates resistant to at least one antibiotic downstream of swine animal agriculture facilities than downstream of row crop farms. While this difference in *Salmonella* antimicrobial resistance is an important finding, it is also important to note that the proportion of *Salmonella* isolates resistant to antibiotics in upstream samples was also statistically significantly different (p value = 0.0057) between swine agriculture facilities and row crop farms. Furthermore, as mentioned previously, there was no difference between incidence of antibiotic resistant bacteria upstream and downstream of the animal agriculture facilities. Therefore, while the two downstream samples have different proportions of *Salmonella* isolates with resistance to at least one antibiotic, it cannot be concluded that this difference is attributable to demonstrable impacts of the swine animal agriculture or row crop farms on the streams of the study.

Human Isolates

People over the age of 18 who lived within one mile of a study farm were recruited to participate in this study. A total of 126 people were enrolled in the study and

were asked to submit fecal samples to the WFUBMC laboratory once a month for 12 months. A total of 578 fecal specimens were received over the duration of the study. This was much lower than the total number of specimens anticipated (see Chapter 6).

As previously indicated, once the specimens were received at WFUBMC, they were screened for *Enterococcus* sp., *E. coli* and S*almonella* sp. using low levels of selective antibiotics to exclude excessive isolation of antibiotic-sensitive enteric bacteria. From the 578 specimens submitted, no *Salmonella* isolates were obtained. No *Enterococcus* sp or *E. coli* with at least minimal resistance to the screening drugs were detected in 285 (49%) of the fecal specimens There were 106 specimens (18%) that had at least one minimally resistant *E. coli* and 200 specimens (34.5%) that had at least one minimally resistant *Enterococcus* sp.. There were some instances in which both minimally resistant *E. coli* and *Enterococcus* were isolated from a single specimen, as well as instances in which more than one isolate of each target genera/species were present. There were a total of 148 biochemically confirmed *E. coli* isolates and 265 confirmed *Enterococcus* sp. isolates collected from human specimens.

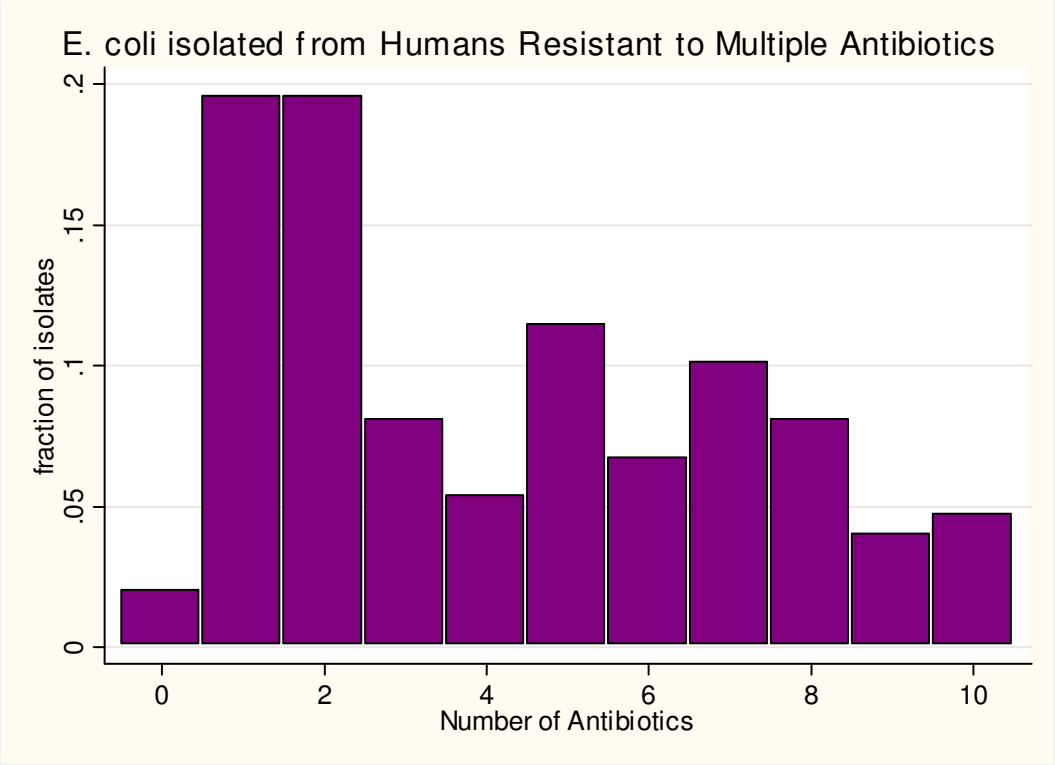
Human E. coli Isolates

Occurrence of Resistance

Of the 148 *E. coli* isolates from stool samples, only 2% (3 isolates) were not resistance to any of the 15 antibiotics studied at the established MIC breakpoints. Seventy-eight percent of the human *E. coli* isolates were resistant to 2 or more antibiotics, and nearly 5% (7 isolates) were resistant to 10 different antibiotics (figure 5.22). As also

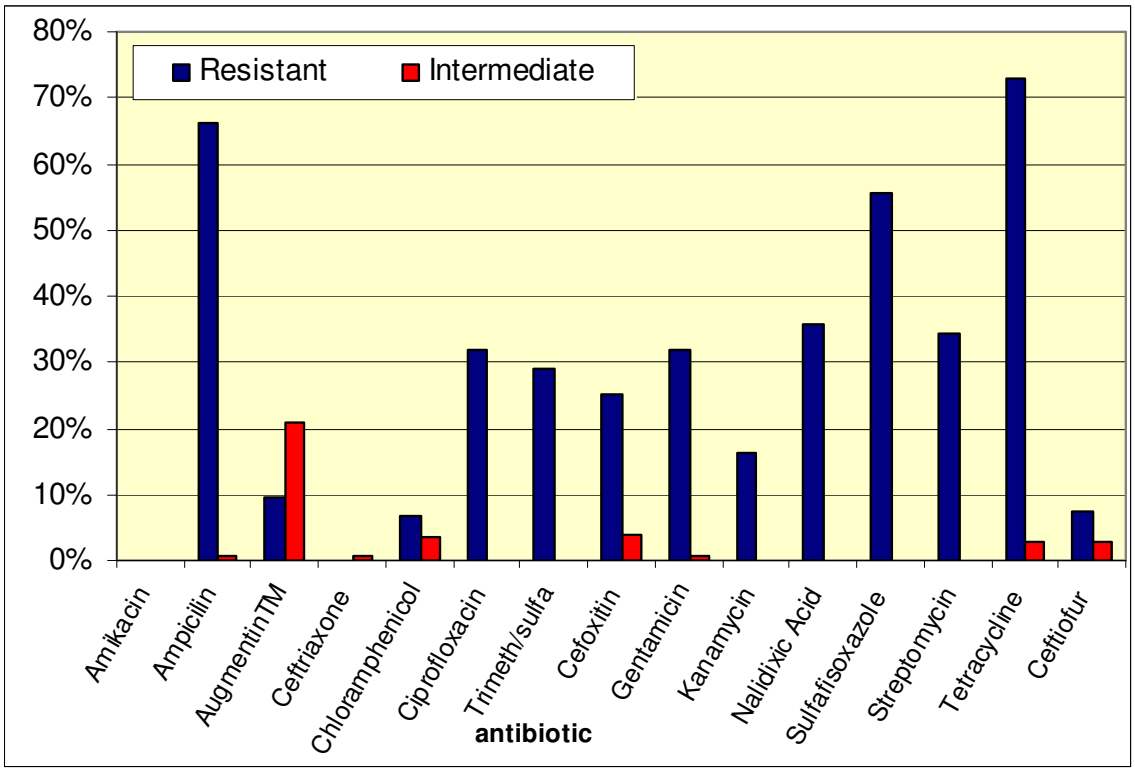
seen in the environmental isolates, resistance to tetracycline was most prevalent among human *E. coli*, with 73% of isolates resistant.

Figure 5.22: Fraction of *E. coli* Isolates from Human Study Participants Resistant to Different Numbers of Antibiotics (n=148)



Also similar to the environmental isolates, human isolate resistance to ampicillin and sulfisoxazole were the second and third most prevalent with 66% and 55% being resistant to these drugs, respectively. Additionally, greater than 30% of isolates were also resistant to gentamicin, streptomycin, naladixic acid and ciprofloxacin (figure 5.23). High incidence of ciprofloxacin resistance in human fecal isolates is notable, as this was one of the drugs for which no resistance was found in *E. coli* isolates from any of the environmental samples. There were no human *E. coli* isolates resistant to amikacin (as seen in environmental isolates), nor were there human isolates resistant ceftriaxone.

Figure 5.23: Percent of Total Human Subject *E. coli* Fecal Isolates Resistant to the Various Study Antibiotics (n=148)



When comparing the occurrence of resistance to the various drugs between *E. coli* isolates from human and environmental samples, it is important to recall that the human isolates were pre-screened with antibiotics for isolation prior to subsequent antibiotic resistance testing. Forty-nine percent of fecal samples yielded no minimally resistant bacteria at all and 81.7% yielded no minimally resistant *E. coli*. However, when calculating the percent resistance based upon specimen number (adjusting for cases in which there were multiple isolates from a single specimen) rather than individual isolates, there is still greater than 10% incidence of tetracycline (17.4%), ampicillin (15.8%) and sulfisoxazole (13.2%) resistance.

Patterns of Antibiotic Resistance in Human E. coli Isolates

Tetracycline resistance was the most prevalent among the all bacterial isolates. Therefore, it was not unexpected that most multi-drug resistant bacteria were resistant to tetracycline. In the environmental samples, all but one of the 127 *E. coli* and *Salmonella* isolates with resistance to four or more antibiotics were resistant to tetracycline. With the human isolates however, this is not the case. Ampicillin resistance was most prevalent among the multi-drug resistant human fecal *E. coli* isolates, with 97% (73/75) of them ampicillin-resistant. By comparison, 57% (43/75) of the human *E. coli* isolates resistant to four or more antibiotics were resistant to tetracycline. Furthermore, with increasing number of antibiotics to which an isolate was resistant, the percentage with tetracycline resistance was actually lower. Only 38% (19/50) of the isolates resistant to six or more antibiotics were resistant to tetracycline. In contrast, 89 of the 95 isolates (94%) that were resistant to 5 antibiotics or **fewer** had resistance to tetracycline.

Ninety-seven percent (28/29) of the human *E. coli* isolates resistant to only one antibiotic were resistant to tetracycline. The remaining 1 isolate was resistant to sulfisoxazole. Of those resistant to 2 antibiotics, 18 isolates (62%) were resistant to tetracycline and ampicillin, 9 (31%) were resistant to tetracycline and sulfisoxazole, 1 (3%) was resistant to tetracycline and trimethoprim/sulfamethoxazole and 1 isolate (3%) was resistant to ciprofloxacin and naladixic acid.

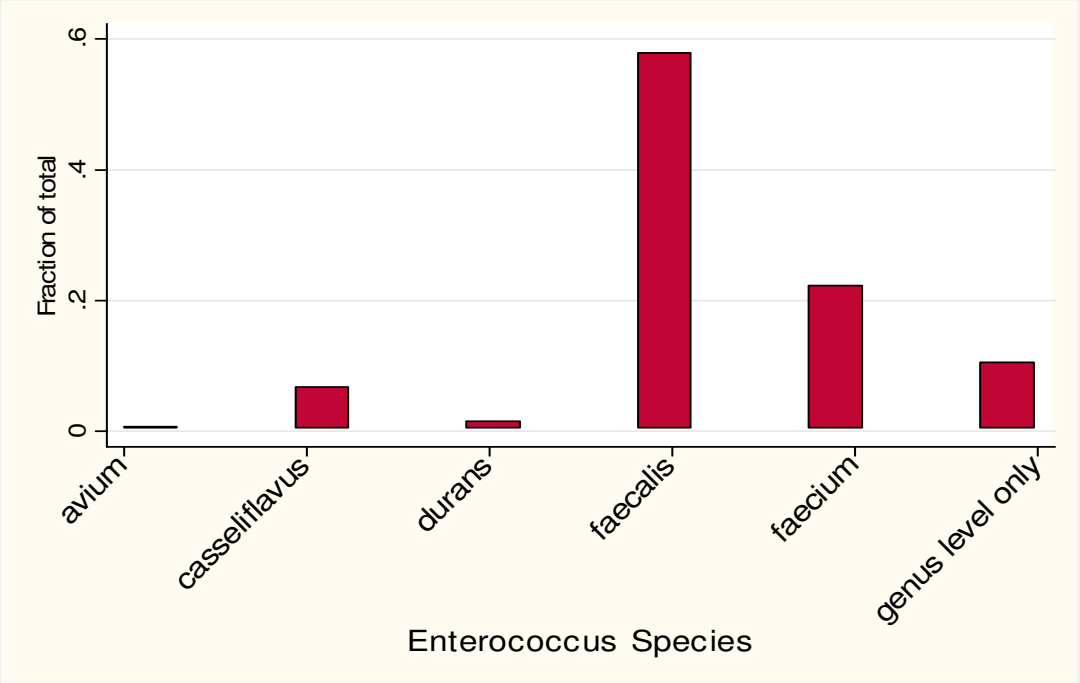
Unlike the environmental *E. coli* isolates, there were no predominant patterns of multiple antibiotic resistance among the human *E. coli* isolates. Among the 74 isolates (50% of the human *E. coli*) resistant to 3 to 8 antibiotics, each had a different combination of antibiotics to which they were resistant (Appendix A-table1). There was

a pattern of multiple resistance however, among the 13 isolates resistant to 9 and 10 antibiotics. All 13 were resistant to the following 9 antibiotics: ampicillin, sulfisoxazole, naladixic acid, kanamycin, gentamicin, ciprofloxacin, streptomycin, trimethoprim/sulfamethoxazole, and cefoxitin; and of the 7 isolates resistant to 10 antibiotics 2 include resistance to tetracycline, 3 are resistant to ampicillin/clavulanic acid (Augmentin™), 1 is resistant to chloramphenicol and 1 is resistant to ceftiofur.

Human Enterococcus sp. Isolates

Enterococcus sp. resistant to at least one of the prescreening drugs were isolated from 34.5% of the total stool specimens received. There were a total 265 Enterococci isolates collected and archived from 200 different human specimens. Of these isolates, *E. faecalis* was the most common species present, representing 57.7% (153/265) of the isolates. *E. faecium* also represented a relatively large fraction of the isolates, 22.3% (59/265) (Figure 5.24). Almost 7% of the isolates were identified as *E. casseliflavus*, and a small fraction were identified as *E. durans* and *E. avium*. There were approximately 10% of the isolates that were only identified to the genus level and could not be speciated. For all of the isolates only confirmed to the genus level, one of the possible candidate species were *E. faecalis* or *E. faecium*; other potential species included *E. gallinarum*, *E. durans* and *E. avium*.

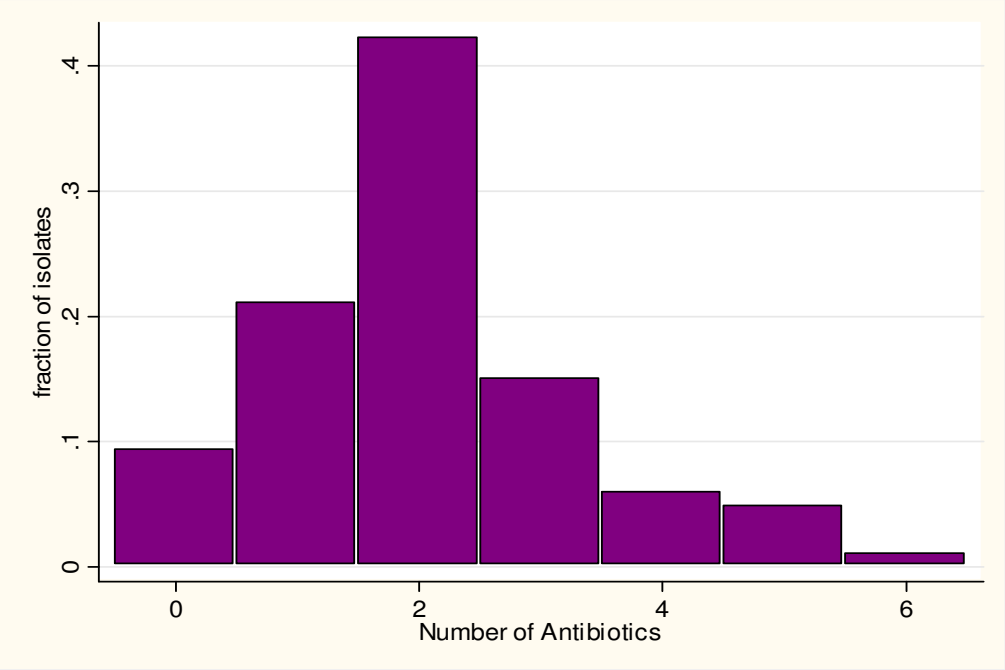
Figure 5.24: Distribution of Different Enterococcus Species Isolates from Human Stool Samples



Occurrence of Resistance to Human Clinically Significant Antibiotics

Multi-drug resistance was common among the human *Enterococcus* isolates. Approximately 60% of the isolates were resistant to two or more clinically significant antibiotics (figure 5.25).

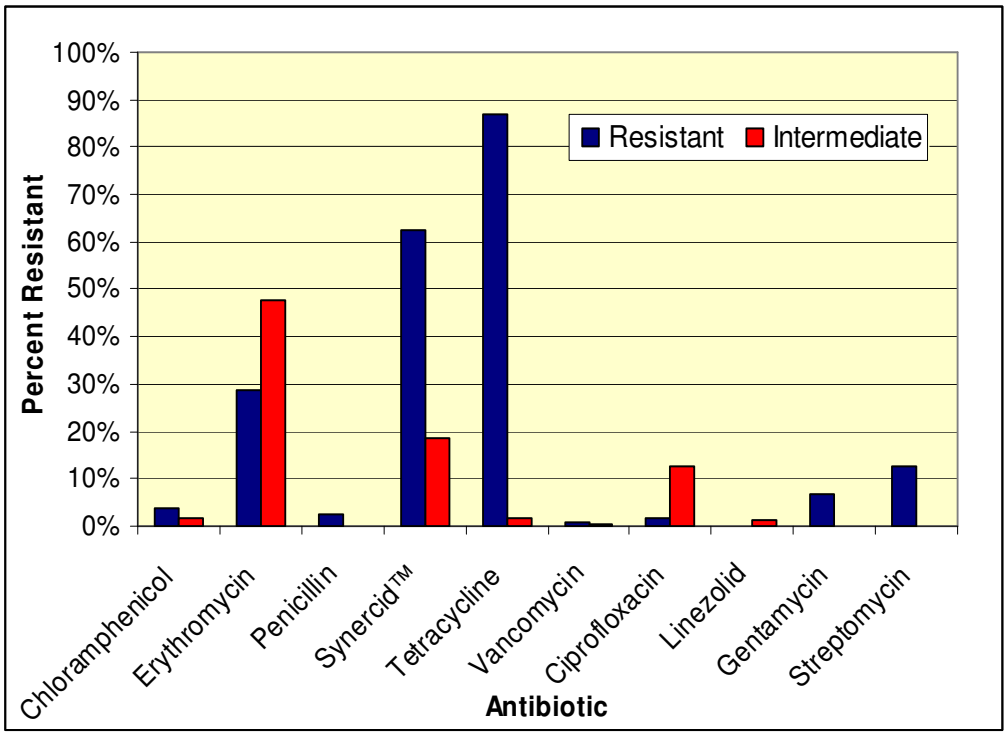
Figure 5.25: Fraction of Total Enterococcus Isolates Collected from Human Stool Samples Resistant to Different Numbers of Clinically Significant Antibiotics



Of the 265 *Enterococcus* sp. isolates from human stool samples, resistance to tetracycline was the most prevalent with 87% of the isolates having resistance. Resistance to quinuprisitn/dalfopristin and erythromycin was also prevalent with 63% and 29% of the isolates, respectively, demonstrating resistance to these drugs.

Linezolid is the only clinically significant drug for which there were no isolates with resistance. However, there was a small fraction of isolates that had resistance to intermediate concentrations of this drug. As with the environmental *Enterococcus* sp. isolates, few human *Enterococcus* sp. isolates were resistant to vancomycin, only two of them. Ciprofloxacin resistance was present in a small percentage (2%) of human *Enterococcus* sp. isolates, and another 12% had resistance to intermediate concentrations of the drug (figure 5.26).

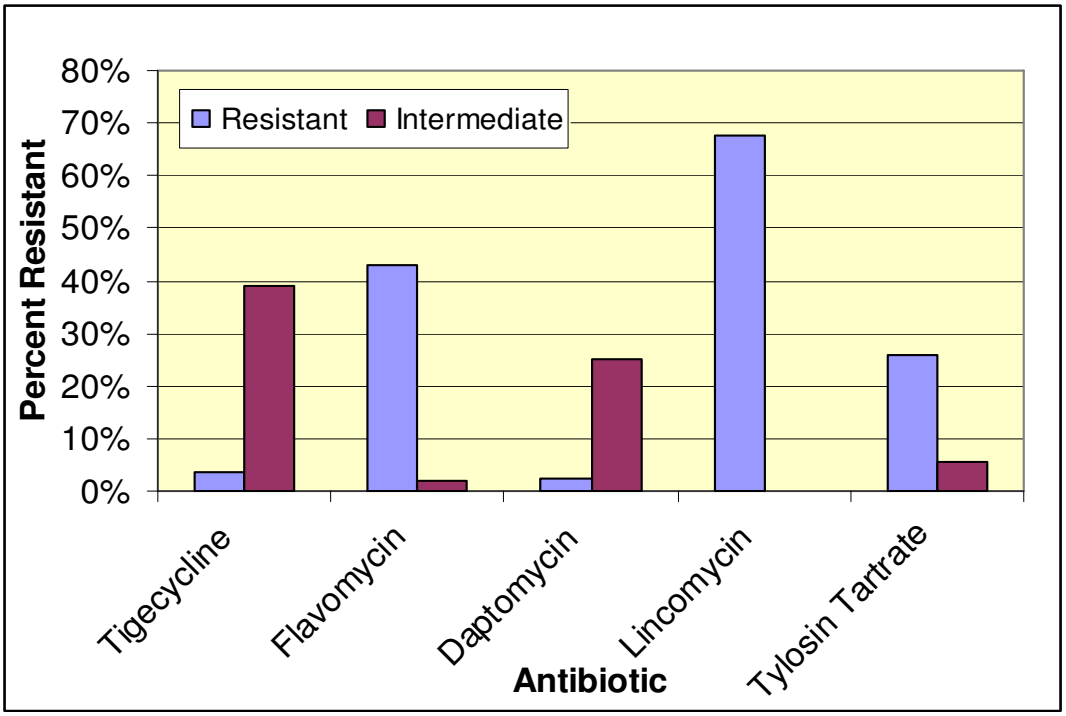
Figure 5.26: Percent Human Enterococcus Isolates Resistant to Clinically Significant Antibiotics



Occurrence of Resistance to Veterinary Antibiotics

Analyzing human *Enterococcus* isolates for resistance to antibiotics of veterinary significance reveals that resistance to lincomycin, flavomycin and tylosin tartrate was frequent, with 68%, 43% and 26% ,respectively, of the human *Enterococcus* isolates collected having resistance to these drugs (figure 5.27). Resistance to tigecycline and daptomycin was much lower, with frequencies of 3% and 2%, respectively.

Figure 5.27: Frequency of Total Human Enterococcus Isolates Resistant to Antibiotics of Veterinary Significance



Antibiotic Resistance by the Most Prevalent Enterococcus Species: E. faecalis and E. faecium

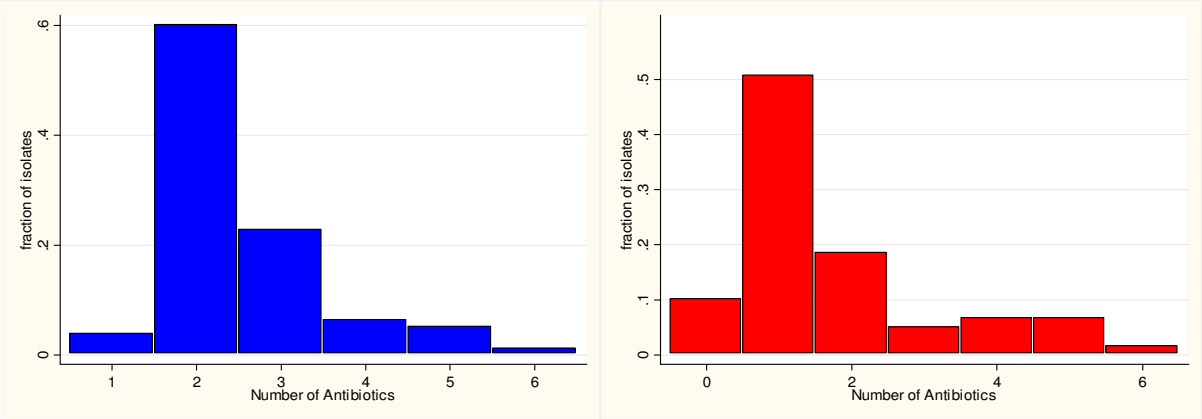
As with the *Enterococcus* isolated collected from environmental samples, the most prevalent species of *Enterococcus* isolated from human stool samples were *E. faecalis* (58%) and *E. faecium(* 22%). Analyzing single and multi-drug resistance in these two species reveals distinct, and statistically different, distributions in the two species (Kolmogrov Smirnov test $p < 0.0001$).

All of the *E. faecalis* isolates were resistant to at least one antibiotic of human clinical significance, and less than 5% of these isolates (6 isolates) have resistance to only one antibiotic (figure 5.28) *E. faecalis* with resistance to two antibiotics was most frequent (60%) and resistance to 3, 4, 5 or 6 antibiotics was progressively less frequent.

For *E. faecium* (figure 5.28b), a larger percentage of the isolates were not multi-drug resistant. Approximately 51% (30 isolates) of *E. faecium* isolates were only

resistant to one drug, and 9.4% (6 isolates) had no resistance at all. Resistance to 4 or more antibiotics was similar in both species as well as in the overall *Enterococcus* sp. with 13% of *E. faecalis* and 15% of *E. faecium* resistant to 4 or more drugs (binomial proportion test p value (0.6786).

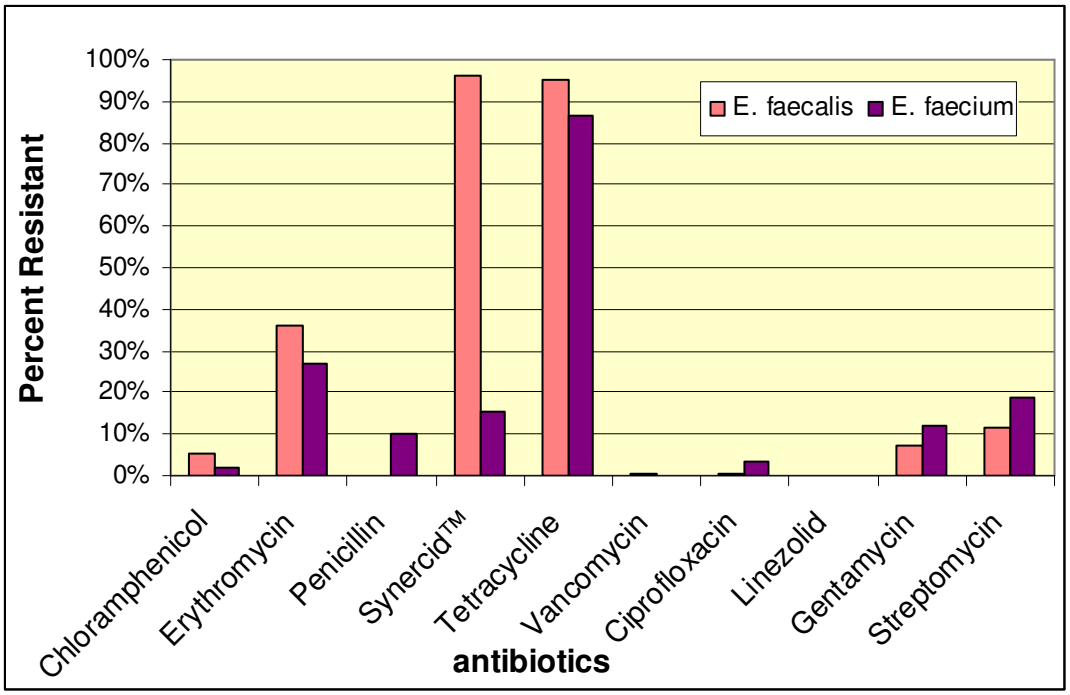
Figure 5.28a and b: Fraction of *E. faecalis*(a) and *E. faecium* (b) isolated from Human Stool Samples Resistant to Different Numbers of Clinically Significant Antibiotics



a) *E. faecalis* **b) *E. faecium***

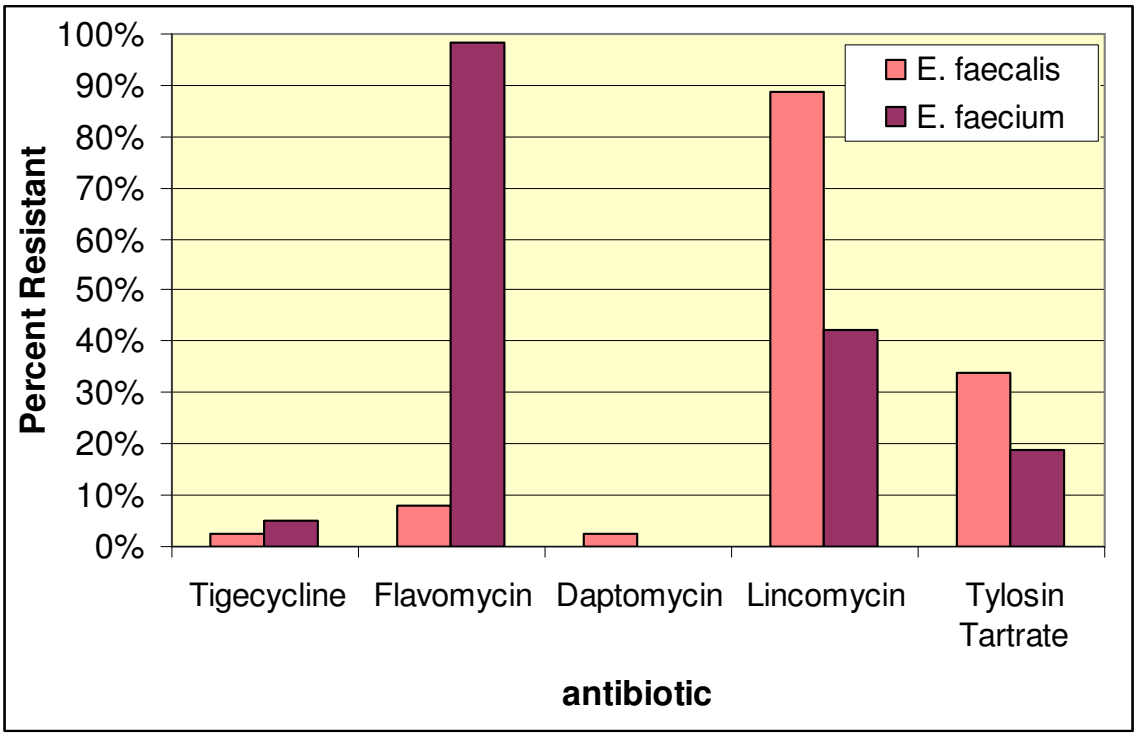
When human *Enterococcus* isolates resistant to various drugs was examined by species, commonalities and notable differences were revealed. An evident difference was in resistance to quinuprisitn/dalfopristin . quinuprisitn/dalfopristin resistance was nearly 100% among *E. faecalis* isolates but only 15% in *E. faecium* isolates. Penicillin resistance also differed between species, with no *E. faecalis* isolates resistant but 10% of *E. faecium* isolates resistant (figure 5.29).

Figure 5.29: Frequency of Human Stool Isolate *E. faecalis* and *E. faecium* Resistance to Various Human Clinically Significant Antibiotics



Analysis of resistance to veterinary drugs in human *Enterococcus* isolates by species revealed flavomycin resistance in almost 100% of the human *E. faecium* isolates but in less than 10% of the human *E. faecalis* isolates. Using binomial proportion analysis, this difference is considered statistically significant ($p < 0.0001$). There were also differences in resistance among these two predominant *Enterococcus* species of human isolates with regard to lincomycin and tylosin tartrate ($p < 0.0001$ and $p = 0.0285$, respectively). Of the *E. faecalis* isolates, 89% and 34% were resistant to lincomycin and tylosin tartrate, respectively, while only 42% and 18% of the *E. faecium* isolates were resistant to these drugs (figure 5.30). There were no significant differences in the proportion of human *Enterococcus* isolates resistant to daptomycin or tigecycline by species ($p = 0.2099$ and $p = 0.3670$).

Figure 5.30: Frequency of Human *E. faecalis* and *E. faecium* Resistance to Various Antibiotics of Veterinary Significance



Patterns of Single and Multi-drug Resistance in Human Enterococci Isolates

Human stool *Enterococcus* isolates were resistant to several different antibiotics important in clinical medicine, as was found for environmental isolates. Among human *Enterococcus* isolates, tetracycline resistance was the most frequent. Tetracycline resistance was 89% for *Enterococcus* sp. resistant to only one drug, and >95% for those resistant to two or more drugs. quinuprisitn/dalfopristin and erythromycin resistance was also common among the multi-drug resistant isolates. Of the 72 isolates resistant to three or more clinically significant antibiotics, all but 2 were resistant to erythromycin and all but 9 were resistant to quinuprisitn/dalfopristin. High level aminoglycoside resistance was prevalent among those *Enterococcus* sp. isolates resistant to four or more drugs, with

all but one isolate of the 32 total, resistant to high levels of streptomycin or gentamicin or both. Though less frequent, there were also some enterococci resistant to chloramphenicol (4%), ciprofloxacin (2%), vancomycin (1%) and penicillin 3%). One isolate was of potential clinical concern because it had resistance to vancomycin and ciprofloxacin, as well as tetracycline.

For the veterinary drugs, there were three antibiotics to which many of the human Enterococcus sp. isolates were resistant: Lincomycin (the most prevalent at 68%), flavomycin (43%) and tylosin tartrate (26%). Of the total human *Enterococcus* sp. isolates, 6 (2%) were resistant to daptomycin and 9 (3%) were resistant to tigecycline.

When examining human *Enterococcus* sp. resistance to combinations of veterinary drugs, there were 18 isolates that had resistance to all three of the veterinary drugs mentioned above. Among human *Enterococcus* sp. isolates, all had resistance to at least one of the five veterinary drugs, but none had resistance to all five or even 4 of the 5 drugs.

For the combined veterinary and human clinically significant antibiotics, multiple resistance in enterococci was the norm rather than the exception (see Appendix A for complete list of profiles). Of the 265 human *Enterococcus* sp. isolates, only one lacked resistance to any of the drugs tested and only 26 (10%) had resistance to only one drug. Resistance to three antibiotics was most frequent at 35%; nearly 20% were resistant to two antibiotics, nearly 13% were resistant to 5 antibiotics and nearly 15% were resistant to 6 or more antibiotics. These percentages of drug resistance in human *Enterococcus* sp. isolates were different from those of the environmental isolates, which had higher proportions of isolates resistant to six or more antibiotics, at 35% compared to 15% of the

human isolates. These findings for resistance patterns in human enterococci were different from the antibiotic resistance patterns found in the Gram-negative bacteria. With *E. coli* and *Salmonella*, there was overall more resistance among the human isolates than the environmental isolates.

A notable finding was the relatively high frequency of human *Enterococcus* sp. isolate resistance to tylosin tartrate (26%). In comparison, almost 50% environmental Enterococcus isolates also were resistant to this drug, mostly isolates from swine waste samples. Comparatively few of the isolates found in cattle manure or in stream water samples were resistant to this drug. Furthermore, there did not seem to be intrinsic resistance to this drug by Enterococcus species.

Comparative Resistance

The above analyses examined the occurrence and frequency of antibiotic resistance in the environment and in people who live in the rural eastern North Carolina communities participating in the study. To understand the potential impact of animal agriculture on people in these communities, it was important to analyze the frequency of resistance in the two exposure groups studied: 1) those that live near or work on animal agriculture facilities (exposed) and 2) those that live near or work on row crop farms (unexposed). Furthermore, it was important to take a closer look at the frequency of resistance found in the bacterial isolates collected from the human stool samples compared with those collected from the environment and assess for similarities and differences.

Analyses of Human Antibiotic Resistance by Exposure Group

There were 87 people who submitted fecal samples for this study. Of those people, 40 (46%) were associated with row crop farms and 47 (54%) were associated with CAFOs.

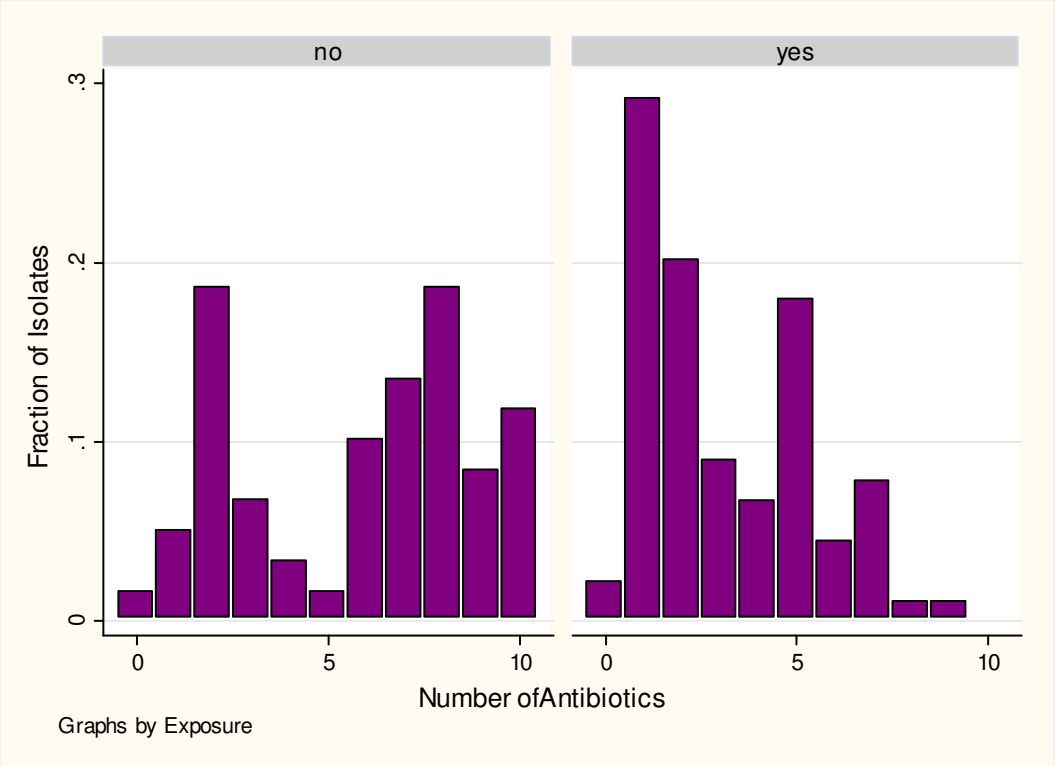
E. coli Isolates

Resistant *E. coli* isolates were collected from people who lived near or worked on CAFOs as well as those associated with row crop farms. Of the 148 isolates collected from the stool samples, 89 of them (60%) were isolated from people in the animal agriculture communities and 59 isolates (40%) were collected from people associated with the row crop farms.

E. coli isolates collected from those people who live near or work on row crop farms (no exposure) had a statistically different frequency distribution of multi-drug resistance than the *E. coli* isolates collected from people associated with swine CAFOs (Kolmogrov-Smirnov $p < 0.0001$) (figure 5.31). Overall, isolates collected from people associated with row crop farms have a higher frequency of multi-drug resistance ($p = 0.004$) as well as a higher proportion of isolates resistance to higher numbers of antibiotics. The highest number of antibiotics to which *E. coli* isolate had resistance was 9 for people associated with CAFOs and 10 for people in row crop communities. When comparing proportions of the two exposure groups for *E. coli* resistance to 4 or more antibiotics and 6 or more antibiotics, the proportion of isolates collected from people in row crop communities had higher frequencies of resistance to the multiple drugs than those isolates collected from CAFO communities ($p = 0.0007$ & $p < 0.0001$) . In other words, *E. coli* isolates collected from people in row crop communities had resistance to

more antibiotics and at a higher frequency than those *E. coli* isolates collected from people in animal agriculture communities.

Figure 5.31: Fraction of *E. coli* Isolates from Human Study Participants Resistant to Different Numbers of Antibiotics by Exposure group (unexposed n =59 exposed n =89)



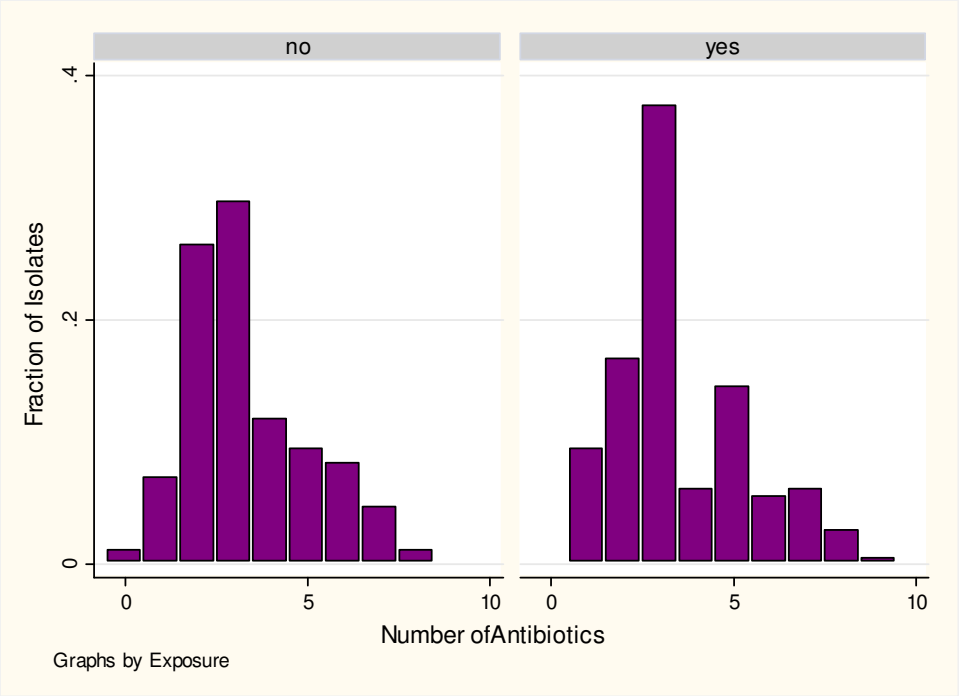
Enterococcus sp Isolates

As with the *E. coli* isolates, *Enterococcus* isolates were collected from stool samples submitted by people who lived near or worked on swine CAFOs and row crop farms. A total of 265 *Enterococcus* isolates were collected and further analyzed. Of these isolates, 86 (32%) were isolated from people associated with row crop farms and 179 (68%) were isolated from people associated with swine animal agriculture.

Unlike the *E. coli* isolates, the frequency distributions of drug resistance in *Enterococci* by exposure group (figure 5.32 were not significantly different (Kolmogrov-

Smirnov p = 0.650). Furthermore there were no differences in the proportions of multi-drug resistance. Proportion analyses were conducted to compare frequency of resistance to 2 or more drugs (p = 0.8696) (including human and veterinary drugs), 4 or more drugs (p=0.8897) and 6 or more drugs (p =.8079) in the two exposure groups.

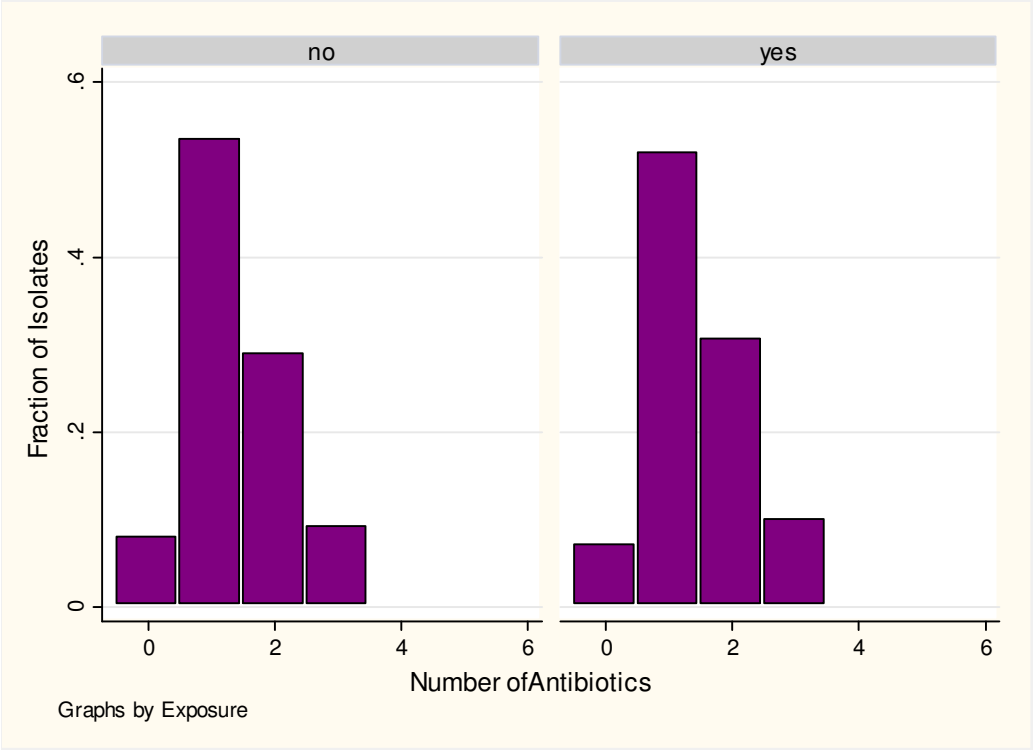
Figure 5.32: Fraction of Enterococcus Isolates from Human Study Participants Resistant to Different Numbers of Antibiotics (including veterinary and human drugs) by Exposure Group (unexposed n =86 exposed n =179)



The above comparisons examined resistance to drugs of veterinary and human significance combined. As impact of animal agriculture on antibiotic resistance in humans is of concern, examining occurrence and frequency of resistance in human isolates to veterinary drugs is important. In this analysis it was revealed that there was no difference in the frequency distributions of resistance to multiple veterinary drugs by

exposure group. The frequency distributions are so similar that the probability of similar distributions was 1.00 (Kolmogrov-Smirnov).

Figure 5.33: Fraction of Enterococcus Isolates from Human Study Participants Resistant to Different Numbers of Veterinary Antibiotics by Exposure Group (unexposed n =86 exposed n =179)



Comparing the proportions of isolates resistant to each of the veterinary drugs independently by exposure, it is revealed that there was no difference in the frequency of resistance to daptomycin (p= 0.3551), tigecycline(0.5048) or tylosin tartrate (p= 0.2105). However, lincomycin resistance was found to be significantly higher in those isolates collected from people associated with swine animal agriculture (p = 0.0469), while flavomycin resistance was significantly higher in isolates collected from people associated with row crop farms (p = 0.0339). As discussed earlier, Enterococcus

resistance to flavomycin and lincomycin are often species dependent. Almost 100% of the *E. faecium* isolates collected from human specimens were resistant to the drug at the MIC_{90} value compared to about 10% resistant *E. faecalis* isolates. Lincomycin resistance was twice as common among *E. faecalis* as in *E. faecium* isolates. As a result, it is possible that the differences in frequency of resistance to these drugs by exposure could be attributed to a difference in the bacterial species collected rather than differences in exposure to these drugs.

There were 153 *E. faecalis* and 59 *E. faecium* isolates collected from human specimens. Of those, 108 (71%) and 36 (61%), respectively, were collected from people associated with animal agriculture while 45 (29%) and 23 (39%) respectively, were collected from people associated with row crop facilities. While the proportions of the isolates in each of these species is consistent in both exposure groups ($p = 0.1809$) the difference in numbers of isolates may have influenced the frequency of resistance to flavomycin and lincomycin in the exposure groups.

Summary

Antibiotic resistant bacteria are found in people associated with animal agriculture and row crop farming. Overall, there was a higher proportion of drug resistant bacteria (both *E. coli* and *Enterococcus*) found in people associated with animal agriculture than those associated with row crop farms. However, when examining the magnitude of the number of antibiotics to which the individual bacteria are resistant, there was no difference among the *Enterococcus* isolates collected from people associated with row crop farms compared with those isolates collected from people associated with swine

CAFOs. In contrast, the *E. coli* isolates collected from people associated with row crop farms had a higher proportion of multi-drug resistant bacteria than the isolates collected from people in swine CAFO communities.

Comparison of Resistance in Isolates from Humans and the Environment

E. coli in Humans and the Environment

Drug resistant *E. coli* were found in 86.5% of animal waste samples, 36 % of ground and surface water samples and 18% of human fecal samples. While there was a higher percentage of resistant *E. coli* in animal waste than in human fecal samples, those isolates collected from human samples had a higher proportion of multi-drug resistance ($p = 0.0015$) . Furthermore, those isolates collected from human samples were resistant to more antibiotics overall than those collected from animal waste samples (figure 5.34). Similarly, comparing multi-drug resistant *E. coli* isolated from ground and surface water samples to those isolated from human specimens (figure 5.35), human *E. coli* isolates had a higher proportion of resistance and were resistant to more drugs overall. Human isolates were resistant to as many as 10 antibiotics (7 isolates), while the maximum number of antibiotics to which *E. coli* isolated from water were resistant was 8 (only one isolate).

Figure 5.34: Frequency Distribution of *E. coli* Isolates Resistant to at least One Antibiotic Collected from Animal Waste (left) and Human Stool Samples (right)

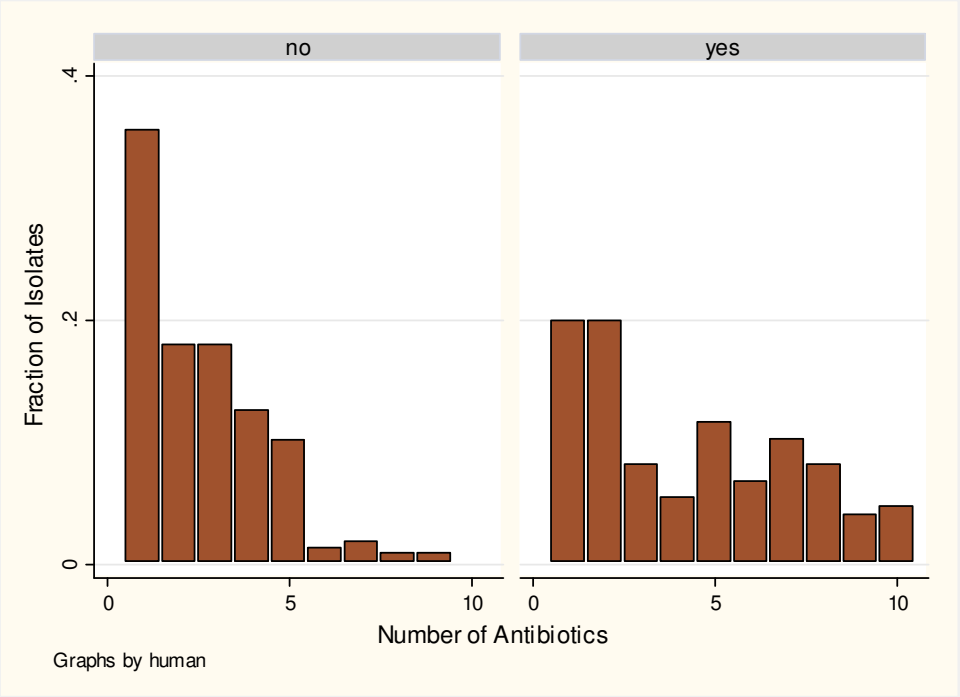
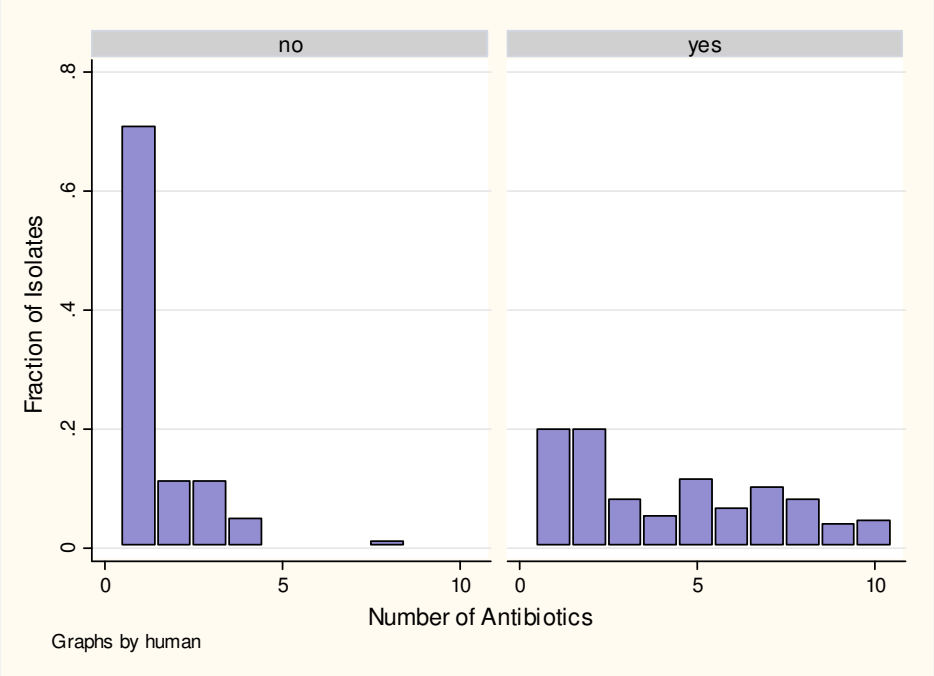


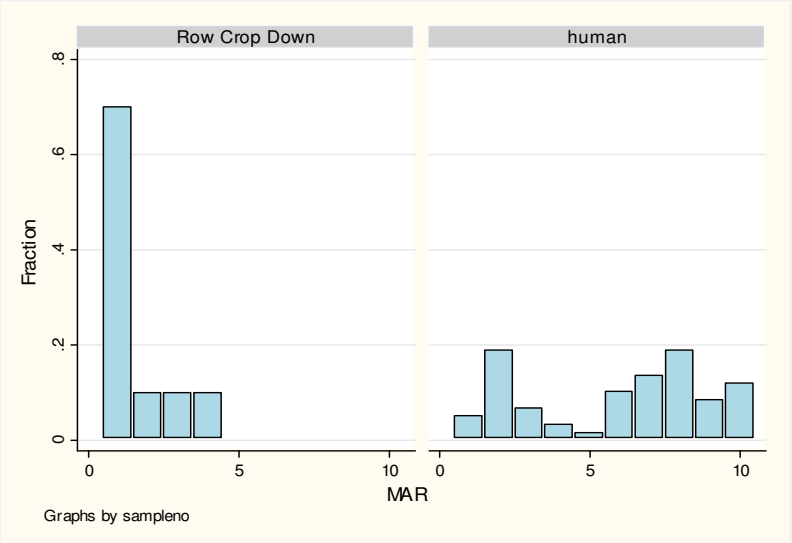
Figure 5.35: Frequency Distribution of *E. coli* Isolates Resistant to at least One Antibiotic Collected from Ground and Surface Water (left) and Human Stool Samples (right)



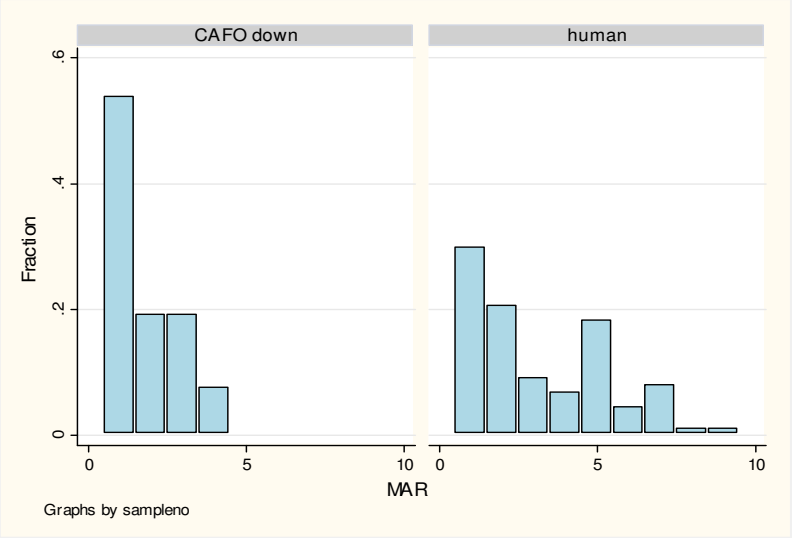
Stratifying the analysis of *E. coli* isolates collected from stream water and human stool samples based upon farm association (figure 5.36a&b) the data reveal that in both types of communities, there was a higher proportion of multi-drug resistance in human isolates than in isolates found in environmental waters (for row crop farms $p < 0.0001$, and for swine CAFOs $p = 0.0250$).

Figure 5.36: Frequency Distribution of *E. coli* Isolates Resistant to at least One Antibiotic Collected from Downstream Water Samples (left) and Human Stool Samples (right) by Farm Type

a. Row Crop



b. CAFO



Enterococcus in Humans and the Environment

In contrast to *E. coli* there was a higher percentage of mono – and multi-drug resistance in environmental and human *Enterococcus* sp. isolates. Thirty-five percent of human fecal samples had *Enterococcus* sp. resistant to at least one antibiotic, and 31% had *Enterococcus* sp. resistant to two or more drugs. In animal waste and environmental water samples, 99% of isolates were resistant to at least one drug and 95% and 93%, respectively, had isolates resistant to two or more drugs.

When analyzing drug resistance in *Enterococcus* from animal waste and human fecal samples (figure 5.37), there was a higher proportion of multi-drug resistance in the animal waste samples ($p = 0.0340$). Enterococci isolated from animal waste were resistant to more antibiotics than the *Enterococcus* isolated from humans. The proportions of *Enterococcus* isolates from animal waste and human sources resistant to 4 or more drugs and 6 or more drugs were significantly different ($p<0.0001$ in both cases).

Comparing multi-drug resistance in *Enterococcus* isolates collected from surface water and humans (figure 5.38), there was no significant difference in the proportion of isolates resistant to 2 or more antibiotics ($p = 0.1031$), 4 or more drugs ($p = 0.9944$) or 6 or more drugs ($p=0.2763$). Furthermore, stratifying the data by farm association and comparing isolates collected downstream of the farms to those isolates collected from human stool samples (figure 5.39 a&b), there was no difference in the proportions (row crop water vs. human $p = 0.6621$, swine CAFO water vs. human $p = 0.9901$).

Figure 5.37: Frequency Distribution of *Enterococci* Isolates Resistant to at least One Antibiotic Collected from Animal Waste (left) and Human Stool Samples (right)

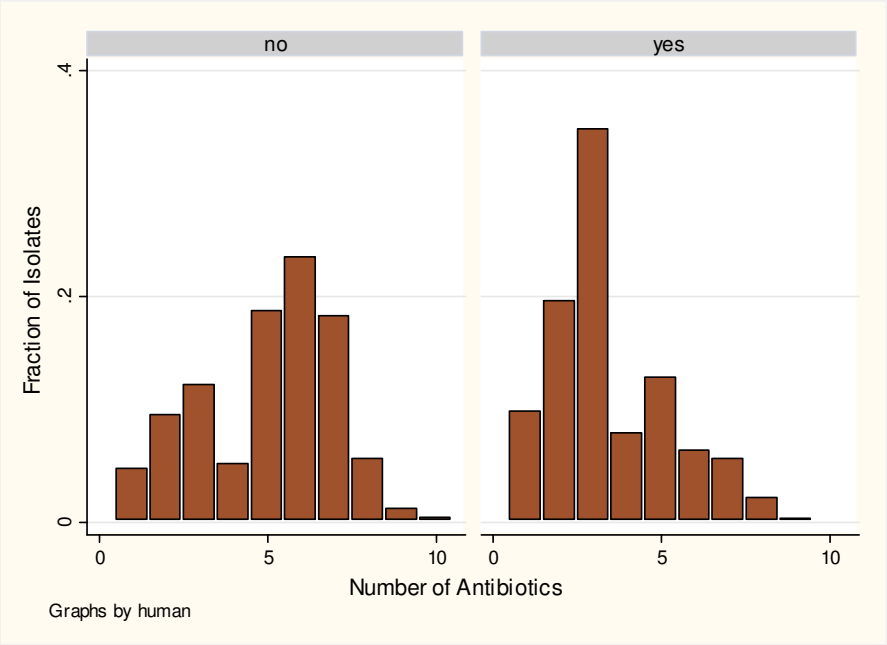


Figure 5.38: Frequency Distribution of *Enterococci* Isolates Resistant to at least One Antibiotic Collected from Ground and Surface Water (left) and Human Stool Samples (right)

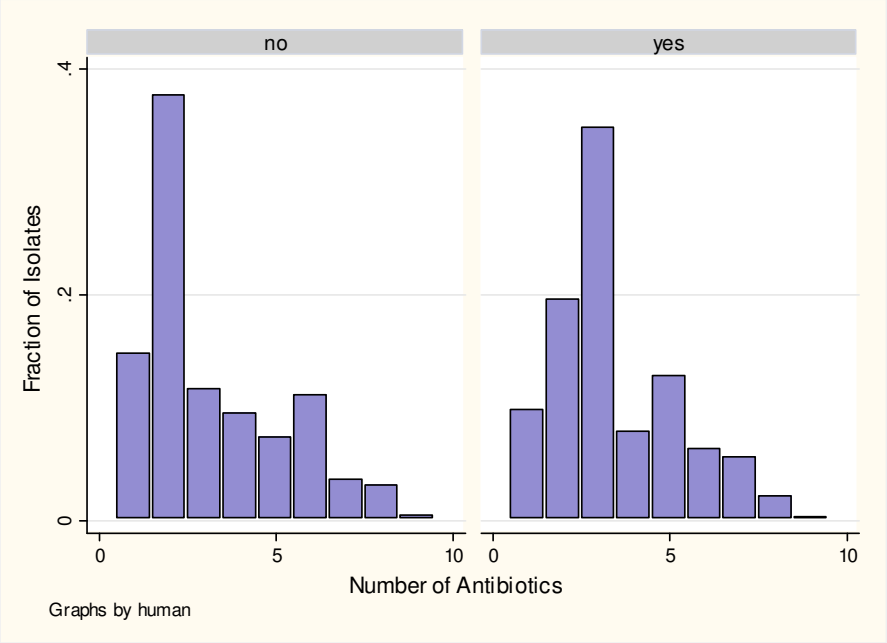
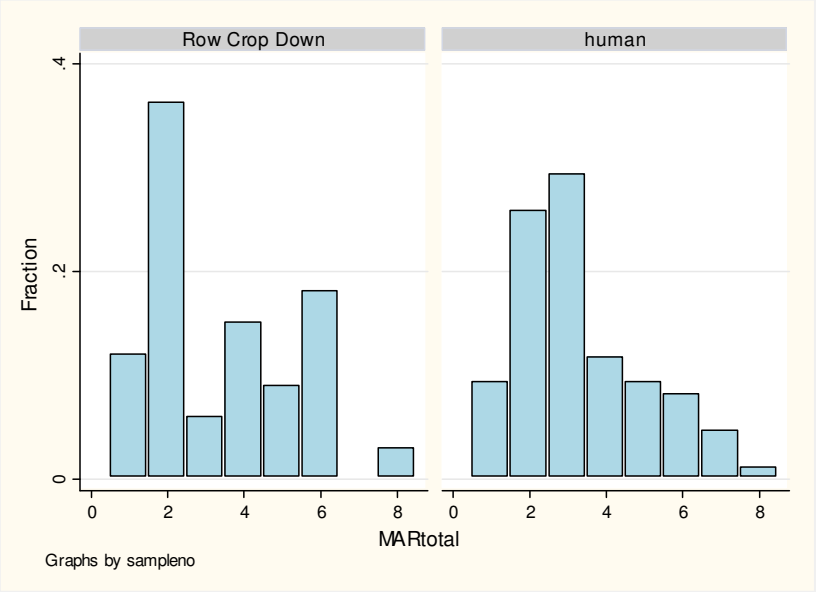
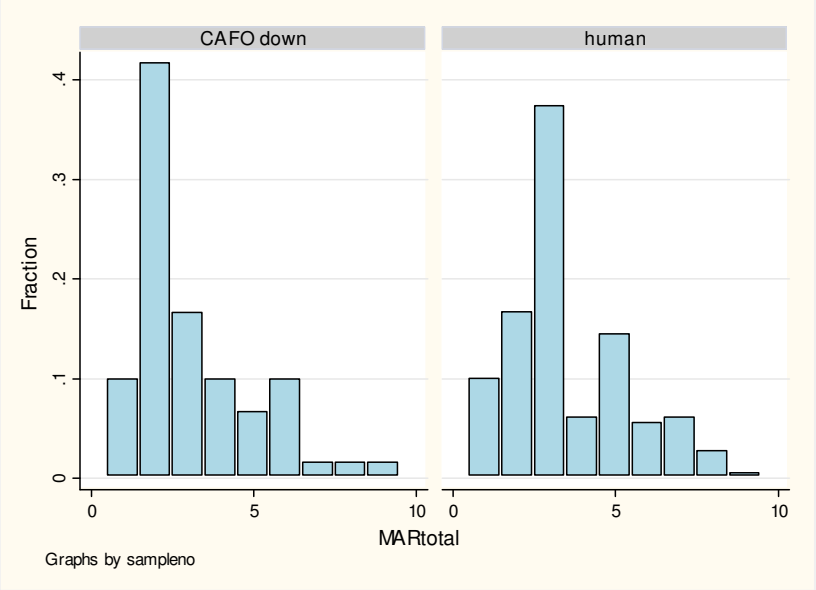


Figure 5.39: Frequency Distribution of *Enterococcus* Isolates Resistant to at least One Antibiotic Collected from Downstream Water Samples (left) and Human Stool Samples (right) by Farm Type

a. Row crop farm



b. CAFOs



Multi-drug Resistance Patterns in Downstream Samples and People

The majority of bacterial isolates collected from water downstream of row crop farms and swine CAFOs were not resistant to any antibiotics. However, there were several bacterial isolates from water that were resistant to multiple drugs.

In water samples collected downstream of animal agriculture facilities, there were seven *E. coli* isolates resistant to 3 or more drugs. Five of these isolates were resistant to 3 antibiotics and there were 4 distinct patterns of resistance (Table 5.9). The remaining two isolates were resistant to four different antibiotics, each resistant to a different combination. Comparing the patterns of resistance in *E. coli* isolated from the water samples to isolates collected from people, three of the patterns seen in environmental *E. coli* were also seen in *E. coli* from human stool samples.

In the samples collected downstream of row crop farms there were 2 *E. coli* isolates with resistance to 3 or more drugs. One was resistant to 3 drugs and the other 4 drugs. In both cases, there were no human *E. coli* isolates that had similar patterns of resistance.

Table 5.9: Resistance patterns in *E. coli* Isolates Collected from Downstream Samples of the Two Farm Types

Farm Type	# of isolates	Resistance Pattern*	Human isolate with similar profile (#†)
Swine CAFO	1	FIS KAN TET	Yes (3)
	1	AMP FIS TET	No
	1	AMP SXT TET	Yes (1)
	2	AMP KAN TET	No
	1	AMP CHL FIS TET	Yes (2)
	1	AMP STR SXT TET	No
Row Crop	1	AMP NAL STR	No
	1	AMP FIS SXT TET	No

† the number of isolates from human samples with the sample profile

*Ampicillin (AMP), Quinupristin/Dalfopristin (AUG), Chloramphenicol (CHL), Ciprofloxacin (CIP), Sulfisoxazole (FIS), Cefoxitin (FOX), Gentamicin (GEN), Kanamycin (KAN), Naladixic Acid (NAL), Streptomycin (STR), Trimethoprim/Sulfamethoxazole (SXT) Ceftiofur (TIO), Tetracycline (TET)

There were many *Enterococcus* sp. multi-drug resistant isolates found in water samples. In water samples collected downstream of swine CAFOs, 29 isolates with resistance to 3 or more antibiotics were isolated. Two were resistant to 3 antibiotics, 5 were resistant to 4 antibiotics, 3 were resistant to 5 antibiotics, 6 were resistant to 6 antibiotics and 1 was resistant to 8 different drugs. Each isolate had a different resistance profile (table 5.10). Downstream of row crop farms, there were 17 isolates collected: 2 were resistant to 3 drugs, 5 resistant to 4 drugs, 3 resistant to 5 drug 6 resistant to six drugs and 1 resistant to eight different drugs. Comparing the patterns of resistance in these environmental *Enterococcus* isolates to patterns found in human *Enterococcus* isolates, there were some patterns that matched. In some cases there were resistance patterns that were found in *Enterococcus* sp. isolates from water downstream of both farms as well as in *Enterococcus* sp. isolates from people.

The most notable resistance pattern was isolates resistant to lincomycin, quinupristin/dalfopristin and tetracycline (LIN SYN TET). There were 71 isolates collected from human specimens with this resistance pattern and it was found in water samples downstream of swine CAFOs. This pattern was also seen in isolates collected from swine waste samples, including lagoon and barn flush samples. While this pattern was not found in *Enterococcus* isolates from water downstream of row crop farms, there were isolates with this pattern collected upstream of row crop farms as well as upstream of swine CAFOs. Furthermore, there was an isolate with this pattern isolated from the irrigation ponds in a row crop facility that is known to NOT have animal agriculture impacts. Therefore, while it appears that *Enterococcus* sp. with this pattern may have originated in swine animal agriculture facilities, a conclusive link to this source can not be established.

Another pattern of resistance common among Enterococcus isolated from human stool samples (27 isolates) was: erythromycin, lincomycin, quinupristin/dalphopristin, tetracycline and tylosin (ERY LIN SYN TET TYLT). In this case, this pattern was found downstream of both row crop and animal agriculture facilities. Furthermore, as previously discussed, resistance to 4 of the 5 antibiotics could be mediated by the same mechanisms. Therefore, using this profile to establish an origin may not be well founded.

Table 5.10: Resistance patterns in *E. coli* Isolates Collected from Downstream Samples of the Two Farm Types

Farm Type	# of isolates	Resistance Pattern[§]	Human isolate with similar profile (#[†])
CAFO	1	CIP LIN SYN*	N
	1	DAP LIN TET	N
	4	LIN SYN TET	Y (71)
	4	LIN SYN TGC	N
	1	CHL LIN SYN TET	N
	1	CIP LIN SYN TET	N
	1	LIN STR SYN TET	N
	1	LIN SYN TET TGC*	Y (3)
	2	FLV LIN SYN TET	Y(8)
	1	ERY FLV LIN TET TYLT	Y(1)
	2	ERY LIN SYN TET TYLT*	Y (27)
	1	CHL ERY LIN SYN TET TYLT	Y (1)
	1	FLV LIN STR SYN TET TYLT	N
	1	ERY LIN STR SYN TET TYLT	Y (6)
	1	ERY LIN SYN TET TGC TYLT*	Y(1)
	2	ERY FLV LIN SYN TET TYLT*	Y(3)
	1	ERY FLV GEN LIN SYN TET TYLT	N
	1	CHL ERY FLV GEN LIN SYN TET TYLT	N
	1	ERY FLV GEN LIN PEN STR SYN TET TYLT	N
Row Crop	1	CIP LIN SYN*	N
	1	LIN SYN TYLT	N
	1	LIN FLV SYN TYLT	N
	1	CIP FLV LIN TET	Y (2)
	1	LIN SYN TET TYLT	Y (1)
	1	LIN SYN TET TGC*	Y (3)
	1	ERY FLV LIN SYN	N
	3	ERY LIN SYN TET TYLT*	Y(27)
	1	ERY FLV LIN SYN TET TYLT*	Y(3)
	1	ERY LIN SYN TET TGC TYLT*	Y(1)
	1	ERY FLV LIN TET TGC TYLT	N
	1	CHL ERY LIN SYN TET TYLT	Y (1)
	1	DAP FLV ERY LIN TET TYLT	N
	1	ERY FLV LIN SYN TET TYLT	Y (3)
	1	CIP ERY FLV LIN STR SYN TET TYLT	N

* Profiles found in downstream samples of both farms

[†] the number of isolates from human samples with the sample profile

[§] Chloramphenicol (CHL), Ciprofloxacin (CIP), Daptomycin (DAP), Erythromycin (ERY), Flavomycin (FLV), Gentamicin (GEN), Lincomycin (LIN), Penicillin (PEN), Streptomycin (STR), Quinupristin/Dalfopristin (SYN), Tetracylcine (TET), Tigecycline (TGC), Tylosin Tartrate (TYLT)

Summary and Conclusions

Mono and Multi-drug resistant enteric bacteria were found in environmental samples, including animal wastes, streams, irrigation ponds and to a lesser extent ground water of rural areas in Eastern North Carolina near farms. Bacteria isolated from animal waste of swine farms had a higher frequency of resistant bacteria than water samples of these farms. Nearly 100% of *E. coli* and *Enterococci*, and 83% of *Salmonella* isolated from swine wastes were resistant to one or more antibiotics. In all surface water samples only 37% and 12% of *E. coli* and *Salmonella* were resistant to any antibiotics while 99% of Enterococci were resistant to at least one drug. As *Enterococcus* species often have some intrinsic resistance to one or more drugs, resistance to multiple drugs in these species were analyzed. Ninety-seven percent of bacteria isolates (including all there genera/species) collected from swine waste samples were resistant to three or more drugs, while only 48% of isolates from environmental water samples were resistant to three or more antibiotics.

Comparing the occurrence and frequency of antibiotic resistance in water samples by proximity to farm types (up and downstream of row crop farms and up and downstream of row crops farms), there were no statistically significant differences in either the occurrence or frequency of the indicator bacteria (*E. coli* and *Enterococci*) at any of the four sampling sites. This includes differences comparing up and downstream samples within farm type (Row crop or swine CAFO) and comparing downstream samples between these two farm types. There was a statistical difference found in the frequency of resistant *Salmonella* isolated downstream of swine CAFOs compared with those isolated downstream of row crop farms ($p = 0.0144$). While this is concerning and

warrants further investigation, it is also important to note that the difference in resistance up and downstream of the swine CAFOs was not statistically significant ($p = 0.4385$) and therefore, the *Salmonella* bacteria found downstream of the animal agriculture facility cannot be conclusively linked to the farm.

In addition to the bacteria isolated from the environment, antibiotic resistant *E. coli* and *Enterococcus* were isolated from stool samples submitted by people who lived near or worked on the study farms. While 49% of the specimens submitted did not contain any resistant bacteria, resistant *Enterococci* were isolated from 35% of the specimens and resistant *E. coli* were isolated from 18% of the specimens.

Eighty-seven people submitted at least one fecal specimen during the course of the study. Of those 40 (46%) were associated with row crop farms and 47 (54%) were associated with CAFOs. Comparing the proportion of bacterial isolates from human samples with antibiotic resistance by farm association, 60% of the resistant *E. coli* and 68% of the resistant *Enterococci* were isolated from people associated with CAFOs.

While the proportion of human specimens with resistant bacteria was higher among those affiliated with animal agriculture, the magnitude of antibiotic resistance in *E. coli* appeared to be greater among those associated with row crop farms. Of all of the *E. coli* isolates collected from stool samples of people associated with swine CAFOs the highest number of antibiotics to which any one bacterium was resistant was 9. Those people associated with row crop farms harbored bacteria with resistance to as many as 10 different drugs. Furthermore, comparing of the proportions of *E. coli* resistant to 4 or more antibiotics ($p= 0.0007$) and 6 or more antibiotics ($p <0.0001$) in those isolates collected from people associated with the two different farms reveals that those

associated with row crop farms harbored a higher proportion of multi drug resistant *E. coli* than those associated with row crop farms.

Antibiotic resistant *Enterococcus* isolated from people in these two farm groups was also examined. In these analyses, no statistical differences in the frequencies or the proportions of multi-drug resistance were seen in the two farm groups.

Comparing the *E., coli* isolates from humans to those found in the environment reveals that while the frequency of drug resistant bacteria was lower in humans than in water (12% in human compared with 37% in environmental water), the magnitude of resistance (i.e. the number of antibiotics to which the isolates are resistant) was not. In downstream samples (of both row crop farms and CAFOs) the highest number of antibiotics to which any *E. coli* isolate was resistant was 4. In the human samples, isolates collected from people associated with swine CAFOs had resistance to as many as 9 drugs and isolates from people associated with row crops were resistant to as many as 10 drugs. Furthermore, when analyzing the differences in the proportion of isolates collected from people to the downstream sample of their farm type, *E. coli* isolates from people had a higher proportion of multi-drug resistant than those from downstream waters (for row crop farms $p < 0.0001$, and for swine CAFOs $p = 0.0250$).

For antibiotic resistant *Enterococcus*, as with the *E. coli,* the overall frequency of resistance was lower in human samples (35%) than in water samples (93%). However, unlike *E. coli* there was no difference in the magnitude of resistance comparing *Enterococci* in downstream water samples compared with the isolates collected from people in those communities (row crop water vs. human $p = 0.6621$, swine CAFO water vs. human $p = 0.9901$).

Examining the profiles of the bacterial isolates from water collected downstream of the farms with those isolates collected from people reveals there are some similarities as well as some differences. For *E. coli* isolates resistant to three or more antibiotics collected downstream of swine CAFOs, 3 of the 6 isolates matched antibiotic resistance profiles found in *E. coli* isolated from human specimens. In contrast neither of the two antibiotic resistance profiles found in *E. coli* isolates from water downstream of row crop farms matches a profile in a human isolate. In multi-drug resistant *Enterococcus*, 9 of the 18 profiles if bacteria resistant to three or more drugs collected from water downstream of swine CAFOs matched a resistance profile of bacteria isolated from people. Also, 8 of 15 antibiotic resistance profiles of *Enterococcus* isolated from water downstream of row crop farms matched human *Enterococcus* isolates. Of these matching antibiotic resistance profiles, however, there were 4 which were found in isolates collected downstream waters of both row crop farms and swine CAFOs. Furthermore, some of the antibiotic resistance profiles found either downstream of swine CAFOs or row crop farms may have also been found in other environmental samples. For example, resistance to the three antibiotics lincomycin, quinupristin/dalfopristin and tetracycline was found in bacteria isolated downstream of swine CAFOs, in swine waste samples, as well as in many human isolates. While enterococci with this profile were not found in isolates from water downstream of row crop farms, they were found in upstream water samples of both farm types as well as one isolated from an irrigation pond that had no impact by animal agriculture. Therefore, concluding that the source of bacteria with profile was a swine CAFO may not be founded.

In general, establishing linkages between bacteria found on the farms, in water and in humans using phenotypic antibiotic resistance profiles was not successful. There are several reasons this may have occurred.

First, as discussed in chapter 4, there were high levels of background enteric bacteria in the surface water. The concentrations of indicator bacteria upstream of the farms were not statistically significantly different from the concentrations of these bacteria in downstream water. Furthermore, as explored in this chapter, there were not significant differences in the frequency distributions of drug resistance or the proportions of drug resistance in the enteric bacteria studied by sampling site.

This high background concentration of enteric bacteria may have resulted from the region in which the study was conducted. This study was conducted in Eastern North Carolina. While attempts were made to isolate the row crop farms from the influence of any animal agriculture, there was a relatively high density of animal agriculture operations in this region. Furthermore, often study swine farms were in close proximity to non-study animal agriculture facilities. This may have resulted in an increase of antibiotic resistant enteric bacteria in the environment. With an elevated background of enteric bacteria in ambient waters, small impacts from each of the study farms may have been masked. Furthermore, this study examined only 11 animal agriculture facilities and 6 row crop farms. This limited number of farms in the study may not be an adequate sample of the hundreds of farms that are in the region.

A second reason the establishment of links between bacteria in swine waste, ambient waters and people associated with farms could not be made was that there were other potential sources from which people may have been exposed to antibiotics other

than the water or the farms with which they are associated. This was clearly the case for those individuals associated with row crop farms. In some cases, isolates from people were resistant to more than twice the number of antibiotics found in isolates from environmental waters. Furthermore, there were some drugs for which human isolates had resistance while environmental isolates did not.

Finally establishing links based on antibiotic resistance profiles alone may have been problematic due to the overall high occurrence of resistance to certain antibiotics regardless of selective pressure. High frequencies of bacterial resistance to certain drugs could be the result of several different phenomena: 1) an overall high prevalence of background bacterial resistance to certain drugs such as tetracycline resulting from decades of use of the drug 2) intrinsic resistance to particular drugs in a given bacterial species; or 3) the result of genetic packaging that links resistance genes and/or mechanisms of resistance that are effective against many different drugs or classes or drugs even though the sources of selection for such resistance were not present..

In this study, there were certain antibiotics to which many of the isolates were resistant, regardless of source. Resistance to tetracycline was common in both Gram-positive and Gram-negative bacteria. Seventy-three percent of human *E. coli* and 87% human *Enterococci* were resistant to this drug. In environmental samples, 82% 72% and 84% of *E. coli, Salmonella* and *Enterococcus*, respectively, from animal waste samples were resistant to tetracycline and 19%, 10% and 41% of *E. coli, Salmonella* and *Enterococcus*, isolates, respectively from stream water were resistant. Gram-negative bacteria were also frequently resistant to sulfisoxazole and ampicillin: 19% of all environmental *E. coli* and 55% of human *E. coli* isolates were resistant to sulfisoxazole,

while 19% of environmental *E. coli* and 66% of human *E. coli* were resistant to ampicillin. Enterococcus sp. isolates were frequently resistant to quinupristin/dalfopristin, erythromycin, lincomycin, and tylosin: 63%, 29%, 68% and 26% respectively, in human isolates and 68%, 48%, 85% and 49% respectively in environmental isolates.

Resistance to certain drugs, either intrinsic or acquired, is mediated by various mechanisms. Furthermore, the genes that regulate these mechanisms may be transferred via plasmids, or other modes of transmission, that can contain multiple genes together. In regard to the Gram-negative bacteria, resistance to tetracycline, ampicillin and sulfisoxazole has been linked to plasmids that encodes resistance for all three drugs (Oppegaard, H et al (2001), Herrero, A et al. (2006), Hansen et al (2007), Shehabi A.A. et al. (2006)). With regard to resistance found in *Enterococcus*, links have been made to a gene that mediates resistance to macrolides, lincosamides and Streptogramin B classes of antibiotics. As a result, the presence of this one gene (*erm*(B)), can mediate resistance within the bacteria to drugs in these classes including (but not limited to) erythromycin, lincomycin and tylosin tartrate. Furthermore, there is evidence of intrinsic streptogramin A resistance among some *Enterococcus* species. Almost all *E. faecalis* isolates have been found to have intrinsic resistance to streptogramin A compounds, which includes quinupristin/dalfopristin. Many *E. faecium* have been known to acquire resistance to this class of drugs, with the acquisition of one or multiple genes (Kak, V. and Chow, J.W., 2002). Resistance to multiple antibiotics can be related to specific individual resistance genes that often clustered on plasmids. Hence, it is possible that some of the resistance to each individual drug may be the result of exposure to another drug or environmental

condition, such as the presence of heavy metals (the resistance gene for which may also be on the same genetic element), as opposed to the individual drug itself.

Conclusion

Overall, many antibiotic resistant bacteria with similar antibiotic resistance traits were found in both the environment samples of swine waste and surface waters and in human stool samples from people living on or near both swine farms and row crop farms. Multiple antibiotic resistance was frequently found in human, swine waste and surface water samples of this study. While some bacteria found in the environment have similar antibiotic resistance profiles as those found in the environment, the source of the bacteria was not conclusively linked to the farm to which each individual was associated.

Resistant bacteria were found at high frequencies in the swine waste samples but the frequency of resistant bacteria in surface water is much lower than that in swine waste. As discussed in Chapter 4, the lower concentration of bacteria in water may be indicative that the bacteria originating on the swine CAFOs were not being released into environmental waters at detectable levels. However, as previously mentioned, this study was conducted during "normal" weather conditions. Sampling was not conducted during periods of unusually high rain and/or flooding. Therefore, these farms as a potential source of multi-drug resistant bacteria should not be discounted, as elevated levels of bacteria, including antibiotic resistant bacteria, have been observed during and after such storm events.

Of the antibiotic resistant bacteria that were found in the surface water, no statistically significant differences were found in the proportions of *E. coli* and

Enterococcus isolates with multi-drug resistance up or downstream of the farms. Furthermore, there were no differences in indicator bacteria levels or their antibiotic resistance frequencies in downstream samples of swine and row crop farms. The only statistically significant difference found was a higher proportion of multi-drug resistant *Salmonella* from water downstream of swine CAFOs compared with the *Salmonella* isolated from water downstream of row crop farms. The frequency and proportions of multi-drug resistant *Salmonella* upstream and downstream of the swine CAFOs were not significantly different. However, as *Salmonella* is a frank pathogen that is known to be prevalent in food animals, this increase in resistant *Salmonella* is of some health concern. Furthermore, although *Salmonella* (resistant or not) were not detected in any of the human specimens, the finding of antibiotic resistant Salmonella in ambient waters of farms is still significant and warrants further monitoring.

As this research was charged with identifying any potential risk of acquiring antibiotic resistant bacteria originating in a farm via exposure to contaminated water, the null hypothesis that there is no difference in the prevalence of antibiotic resistant bacteria in stream water when comparing animal to non-animal agriculture must be accepted. However, it is possible that people are acquiring resistant bacteria from the farms via some other routes of exposure that were not investigated or elucidated in this study.

Further molecular characterization of the bacterial isolates may provide a better understanding regarding the antibiotic resistance genes that mediate drug resistance in the different isolates. Knowing the actual genes and/or genetic sequences of the bacterial isolates may provide more insight into the origin of the bacteria found in water and people. And perhaps this information will provide definitive links to the potential source

of the resistant bacteria in humans and in swine waste. However, within the scope of this research that link has not been made. Additional research including other potential routes of transmission of the antibiotic resistant bacteria from animal agriculture facilities to humans should be explored.

Chapter 6- Epidemiologic Analyses

Introduction and Background

Identifying, quantifying and characterizing the presence of enteric pathogens and antibiotic resistant bacteria on farms and in the environment is important in understanding the potential for human health risks associated with these sources of exposure. To assess the actual impact on human health, however, it is important to examine the bacteria that the people actually harbor.

In this study, animal agriculture facilities were identified as a potential source of enteric bacteria, including antibiotic resistant *E. coli*, *Enterococcus* sp and the pathogen *Salmonella*. All of these bacteria were present in animal wastes on the farms in relatively high concentrations. Some of these bacteria were also found in the environmental waters.

A conclusive link of the bacteria on the farms, in the water and in people who live near or work on the study farms (CAFOs and row crop farms) was not established. However, the proportion of people living in CAFO communities had a higher proportion of isolates with antibiotic resistance. As discussed in Chapter 5, there may have been other routes of exposure by which people who live in these communities are exposed to bacteria originating on the farm. Furthermore, there may be additional factors that lead to differences in the proportions of antibiotic resistant bacteria in one community or another. Therefore examining antibiotic resistance in the two farming groups may

provide insight into the potential risks in one type of farming community as compared to another.

A substantial amount of information on occurrence and possible human health risks from antibiotic-resistant enteric bacteria can be gained from the analysis of the stool specimens received in this study. Community acquired antibiotic resistant infections are on the rise. Therefore, any information regarding the occurrence and properties of antibiotic resistant bacteria in different communities is important to understand. Keeping in mind the source of these bacteria may not be possible to substantiate, it is possible to characterize the presence of antibiotic resistance bacteria in people of rural Eastern North Carolina communities; examine possible demographic or geographic factors that may be associated with carriage of these resistant bacteria; and make comparisons on the occurrence, locations and properties of antibiotic resistant bacteria between the two different farm types studied, row crop and CAFOs farms.

Objectives

This study was intended to assess the risk of carriage of antibiotic resistant bacteria as a result of association with animal agriculture facilities as compared with those associated with row crop farms. To this end, demographic information as well as potential sources of exposure to these bacteria outside of the animal facilities were assessed and characterized in the overall population as well as the study population by farm association. Comparisons were then made based upon farm association. Furthermore, as there was significant loss to follow up, demographic information from the total recruited population was compared to the actual study participants to determine

if there were any significant losses of any one particular demographic group. Finally, a risk analysis was conducted to determine if in fact there is an increased risk of carriage of antibiotic resistant enteric bacteria among those who live near or work on CAFOs.

Materials and Methods

This study has institutional IRB approval from Wake Forest University Baptist Medical College (WFUBMC), which is the institution responsible for the human health and clinical aspects of the study. The study was also approved by CDC (the funding source of the project). All recruited participants signed an informed consent form prior to enrollment into the study.

Human participants

Farmers and neighbors of the animal agriculture (swine farm) and non-animal agriculture (row crop farm) facilities in the study were recruited to participate in a prospective cohort study. The recruitment goal was to recruit 200 people in total, divided into 4 groups: swine farmers, row crop farmers, swine farm neighbors and row crop farm neighbors. Those participants associated with the swine farms are considered to be the exposed group and those associated with non-animal agriculture are the unexposed (or control) group.

Upon enrollment into the study, each participant was asked to complete a questionnaire (see appendix B) which included questions regarding occupation, personal antibiotic use, contact with animals, water exposures (e.g. contact with environmental surface water for recreation or work, and source of drinking water) and travel history, as well as other personal information that could potentially impact their acquisition of

antimicrobial resistant bacteria or *Salmonella* infection. Additionally, each participant was asked to submit a fecal sample once monthly for one year; the time period of which was concurrent with the environmental sample collection of that farm area. Participants were also asked to submit additional fecal samples if they become sick with a diarrheal illness during the year of participation. Follow up questionnaires accompanied each fecal sample to ensure there was no change in exposure to antibiotics or resistant bacteria.

Fecal samples were collected using a unique method developed by Dr. Chris Ohl et al. (not yet published) that allows the participant to collect his/her own sample and send it to Wake Forest University Baptist School of Medicine (WFUBMC) for processing (see appendix C). This method consists of using a plastic backed absorbent toilet paper instead of toilet paper. Each participant was asked to soil the absorbent paper with fecal matter (at least the size of a quarter in diameter), place a gauze layer that contained transport medium over the top (Cary-Blair medium; to allow the bacteria to survive until the specimen reaches the laboratory for processing), fold it in half and place in a sealable plastic bag. Then this bag along with the monthly questionnaire is mailed to the WFUBMC laboratory for further processing.

Recruitment of Participants

Participants were recruited from those people over 18 years of age that work on or live near the study farms. If a participant is in the exposed group they must work on a study swine farm or live within one mile of a study swine farm (with more intense recruiting focused on those within a half a mile of the farm). If the participant is in the unexposed group they must work on a study non-animal agriculture farm or live within

one mile of a study non- animal agriculture farm and must be greater than one mile from a study animal facility.

As this is an unmatched prospective cohort study, exposed and unexposed participants are not matched. However, some demographic similarities were expected by restricting the study sites to specific counties in Eastern North Carolina.

Due to varying needs and situations, different recruitment techniques were employed in different communities. In some cases the farmers themselves helped with recruitment by talking to their neighbors and inviting them to attend a community meeting lead by the research team to educate them about the study and enroll the interested individuals. In another community, an ad was placed regarding the study in a quarterly neighborhood newsletter issued by one of the growers. This was followed up by mailing letters to many of the neighbors, using the names and addresses of the neighbors provided by the grower in that community. This letter invited the neighbors to attend an informational meeting in their area. The letters were followed up with phone calls by the research team again inviting them to attend the meeting. At the meeting, the research team educated people about the study and enrolled interested study participates.

In the majority of communities, members of the research team canvassed (door to door) the neighborhoods and personally recruited people from households within the study area. As the numbers of candidate participant households in the study areas were relatively small per neighborhood, it was determined that door to door recruitment was more effective than any other techniques such as mailers, community church meetings, etc. Furthermore, since the goal was to recruit as many people in the area as possible, and recognizing that not all people will agree to participate, all houses in the study area

were included, rather than selecting random houses to approach. In the door to door approach there were some instances in which the study team was asked not to approach certain households. There were various reasons for these requests including ill health of the neighbor and personal conflict between the farmer and the neighbor. These requests were limited in number and therefore, not believed to affect the overall outcome of the study.

While recruitment was generally successful, there were limitations to the approaches used. First, by allowing the farmers in the study to help in the recruitment efforts, either by inviting neighbors to the meetings or by requesting certain households not be asked to participate, there was a potential introduction of bias into the study. While this potential is acknowledge, the research team decided that this bias would be minimal as all participates were still required to meet the study requirements.

A second limitation of the recruitment techniques was time and access to neighbors. During the door to door recruitment of neighbors, the research team was limited to certain days and times of the day due to distance from the institutions of the study team (NC State University, UNC-Chapel Hill, and Wake Forest University Baptist Medical Center). The majority of the recruiting was done in the evenings during the work week. Tuesday and Thursday evenings from about 4pm to 8pm were the major recruiting times. The evening hours were selected as people would be returning from work and also for logistics reasons pertaining to travel abilities the recruiting team. The days were chosen as those most likely to find people at home, as Wednesday evenings are commonly reserved for church in many of these communities, and in general Monday and Friday evenings are less likely for finding people at home or willingness to engage in

talk pertaining to the study. With these specific recruiting times, it is possible that those people that work or have other meetings and events in the evenings were missed. Additionally, many people may not have answered the door as the recruiters were strangers in the community. Furthermore, often it began to get dark early in the evening of some recruiting months and people may have chosen not to answer the door after dark.

Human Specimens/Isolates

As discussed in chapter 5, fecal samples were sent to a clinical microbiology laboratory at Wake Forest University Baptist Medical College under the direction of Prof. Chris Ohl, MD. Upon receipt, specimens were assigned a specimen and laboratory number. The fecal matter was then removed from the absorbent paper by swab or if there was only a small amount of fecal matter present, the soiled portion was cut out. The swab or absorbent paper cut out was then placed into Tryptic Soy Broth (TSB) with 20% glycerol. The samples were then sonicated in a water bath for one minute. Next the samples were vortexed on high speed for at least two minutes. The broth was then transferred into a 5ml polypropylene culture tube and frozen at -80ºC until enough specimens were received to analyze them for antibiotic resistance in batches.

When enough specimens were received, they were analyzed for the same three bacterial genera and species that were analyzed for in the environmental samples (*Salmonella* sp., *E. coli* and *Enterococcus* sp.), as well as *Campylobacter* sp. Campylobacter was of interest due to its importance as a human enteric pathogen. Of the *E. coli* and Enterococcus sp., only those that have some resistance to at least one clinically important antibiotic were of interest. Therefore, prescreening on selective agar

with low concentrations of clinically significant antibiotics was done (table 6.1). Any *E. coli* or Enterococcus sp. that were able to grow on these selective agars were isolated, collected and archived for further analyses.

Table 6.1: Concentrations of Prescreening Antibiotics for Human Bacterial Isolation

For E. coli:	**For Enterococcus**
ciprofloxacin 2ug/ml	ampicillin 8 ug/ml
gentamicin 4 ug/ml	gentamicin 250 ug/ml
norfloxacin 4 ug/ml	streptomycin 250 ug/ml
tetracycline 4 ug/ml	quinupristin/dalphopristin 2 ug/ ml
	vancomycin 8 ug/ml
	tetracycline 4 ug/ml

Salmonella sp. and *Campylobacter* sp. are of interest regardless of their resistance traits as they are frank pathogens and are of human health concern. Therefore, these organisms were screened with selective agar without antibiotics. Any isolates found were collected and archived for further analysis.

All archived isolates were purified (per method discussed in chapter 5) and then biochemically identified using Enterotubes® for Gram-negative bacterial isolates or APi20 strep strips for presumptive *Enterococcus* sp. isolates. Upon positive biochemical identification and species confirmation, those isolates that were positively identified as either *E. coli*, *Salmonella* sp. or Enterococcus sp. were analyzed for antibiotic resistance to the same suite of antibiotics as applied to the environmental sample isolates on previously described Sensititre® plates by Trek Diagnostics™ (see Chapter 5).

Data Analysis

Due to the difficulties in participant recruitment as well as the significant number of people that were lost to follow up, the epidemiologic analyses originally proposed were not possible. There is not enough power with the number of clinical specimens received to fully assess the risk of acquiring antibiotic resistant bacteria from exposures on swine farms as compared with those on row crop farms. Furthermore, as discussed in chapter 5, while there was a very high incidence of single and multiple antibiotic resistance in bacteria isolated from the study population, it was difficult to link the isolates found in the human subjects to those found on the farms, in swine waste or in the environmental waters surrounding the farms. Molecular characterization of the isolates may provide greater insights to the possible links or clonal relationships between human, swine waste and environmental water bacteria resistant to antibiotics. Therefore, in these analyses, risk of carriage of antibiotic resistant bacteria rather than acquisition of the bacteria was assessed.

Data Entry and Recording

All information provided by the participants in the introduction (initial) questionnaire was recorded electronically using EpiInfo®. The database was then imported into STATA® and GraphPad® by Instat™ for further data analysis. Using assigned study numbers, all personal information was linked to specimens and bacterial profiles of those bacteria isolated from the specimens.

The total recruited population was characterized based upon demographic traits and geographic information to help characterize similarities and difference in the two

exposure groups and in the overall population that may impact their potential for carriage of antibiotic resistant bacteria. Additionally, as the study had participant loss to follow up, the study population from which specimens were actually received was also tracked and characterized.

Initial epidemiologic-microbiologic analysis examined the specimens received and scored them as positive or negative based upon bacterial isolation and antibiotic resistance screening. Specifically, if one or more bacterial isolates from a single specimen was found to be resistant to at least one clinically significant antibiotic upon subsequent antibiotic resistance screening for MIC, the specimen was scored positive. If a single individual had at least one specimen that was positive for resistant bacteria, that person was scored to be positive for the outcome (carriage of antibiotic resistant bacteria). Those individuals associated with swine farms (the "exposed" group) were then compared with those associated with row crop farms (the "unexposed" group) to determine if there is a higher risk of harboring antibiotic resistant bacteria in their gut flora (outcome) based upon exposure. Risk ratio analysis was used to quantify the potential influence of swine farms on the outcome. This was done using log- linear regression techniques. Some additional variables such as gender, age and income were also assessed to determine if these were potential confounders or effect measure modifiers. The inclusion of variables as possible confounders was determined based upon a 10% change-in-estimate approach. Each variable was included in the model and the coefficients generated were then compared with those of the crude model. If there was at least a 10% change in the coefficients, the variable was included in the final model.

Results

Recruited Participants

Recruitment of study participants proved to be more difficult than anticipated. This can be attributed in part to the number of people that lived within one mile of the farm. As the study region is a rural, by definition there is a lower population density than suburban or urban settings. Furthermore, many of the study farms had relatively few people living within one mile of the facility. This was especially true for the animal agriculture facilities as they have an overall smaller land area for the farm itself and therefore geographically there is a smaller region circumferential area from which to recruit. Additionally, people may choose to live further away from these kinds of facilities when possible.

Another contributing factor to the difficulties in participant recruitment is the nature of the study. Recruiting individuals for any type of study requiring sustained participation is difficult. Even if someone answers the door, which as mentioned earlier was not always the case, there are many people that will not want to participate. Given the sensitive subject matter of this study and the request for fecal samples, recruiting willing participants was even more challenging. While this study did not require the people to go to a medical facility to submit their fecal samples, many people are hesitant to enroll in a study of this nature when it requires submitting such specimens. Discussing fecal matter and submitting samples is uncomfortable for many people. As a result, there were many people that declined to participate in the study, and in many cases people that agreed to participate were later lost to follow up because they submitted no or few fecal specimens.

. A third obstacle to recruitment was the duration of the study. In some cases farm employees and/or neighbors may only live near or work on the farm for part of the year and therefore would not be suitable for sustained, year-long participation the study. Additionally, even for those who are permanent residents of the area, one year can be a long time to be in a study. Several people indicated that submitting a sample once a month for twelve months was not something to which they could commit. This was further evidenced by the number of people lost to follow up in the study that did agree to participate, based on their compliance with monthly fecal specimen submission. Even with reminder letters to the participants, the majority of people recruited submitted fewer than four specimens total over a 12-month time period.

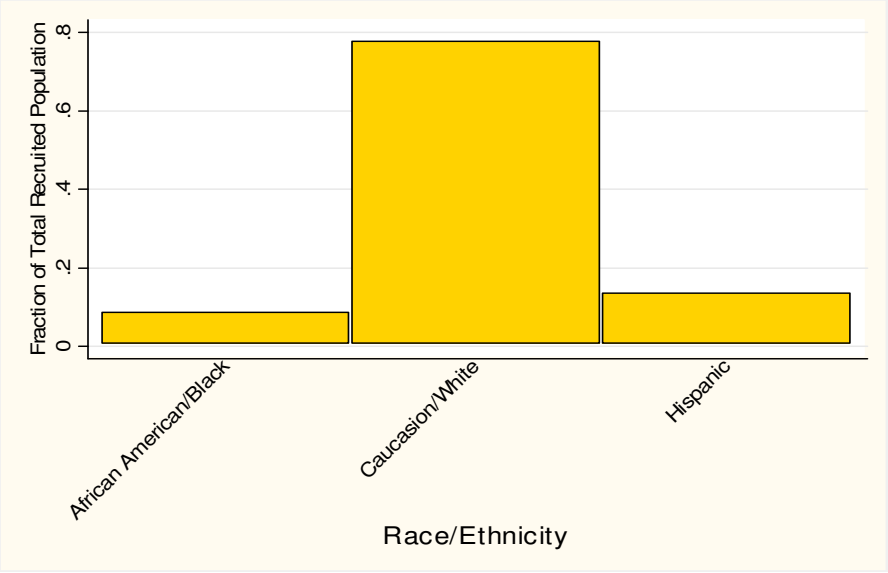
Finally there were instances in which members of the community spoke only Spanish. While efforts were made to translate all study material and recruit in Spanish, explaining the study and recruiting in Spanish by a non-native speaker was often challenging. Having materials in Spanish did not make up for some of the limitations in personal communication and camaraderie that is often required to recruit people into a study. Many of the members of the Spanish speaking communities may have been hesitant to speak with the recruiters. Few members of these communities answered the door, and none of the Spanish speaking neighbors agreed to participate. Those Spanish speakers that did agree to participate were all employees of study farms.

Characterization of Study Participants

Given the aforementioned limitations and difficulties involved in participant recruitment of this study, recruitment was relatively successful. Though the goal of 200

participants was not met, 126 people were recruited into the study. Participants lived in six different Eastern North Carolina Counties including Franklin, Jones, Lenoir, Greene, Pitt and Gates. Overall the enrolled population was well distributed with regard to gender, age, income and occupation. However, with regard to race and ethnicity, the majority of the recruited population was White/Caucasian. Only 9% of the participants were Black/African American and 13% were Hispanic (figure 6. 1).

Figure 6.1: Race/Ethnicity Distribution of Recruited Population

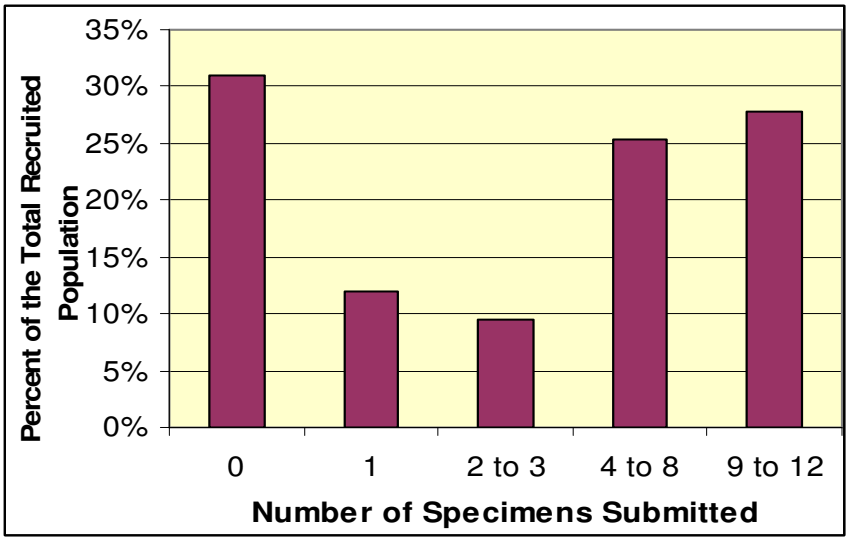


Loss to Follow up

There was significant loss to follow up of the recruited participants. Of the 126 people who were enrolled in the study, only 87 (69%) sent at least one fecal specimen during the study period. And only 15 people (12%) submitted all 12 of the requested specimens. Of the 87 people that submitted at least one specimen, 15 only sent 1

specimen, 12 sent only 2 to 3 specimens, 25 sent 4 to 8 specimens and 35 sent 9 to 12 specimens (figure 6. 2)..

6.2: Percent of Recruited Population that Submitted Different Number of Samples



Even though nearly one third of those recruited failed to submit even one fecal specimen, all of the study farm communities still had at least 5 people who sent at least one specimen and at least 2 people who submitted 4 or more specimens. Some farms had even higher response rates with as many as 10 people submitting 9 or more specimens.

In addition to extent of representation of participants among the study farms, the distribution of representation among animal and non-animal agriculture communities was also of interest. The number of active study participants was not heavily weighted to one farm type or the other, as there was a nearly one to one distribution of the 87 participants, with 47 of them living near or working on an animal agriculture farms and 40 of them associated with row crop facilities.

Participant Demographics

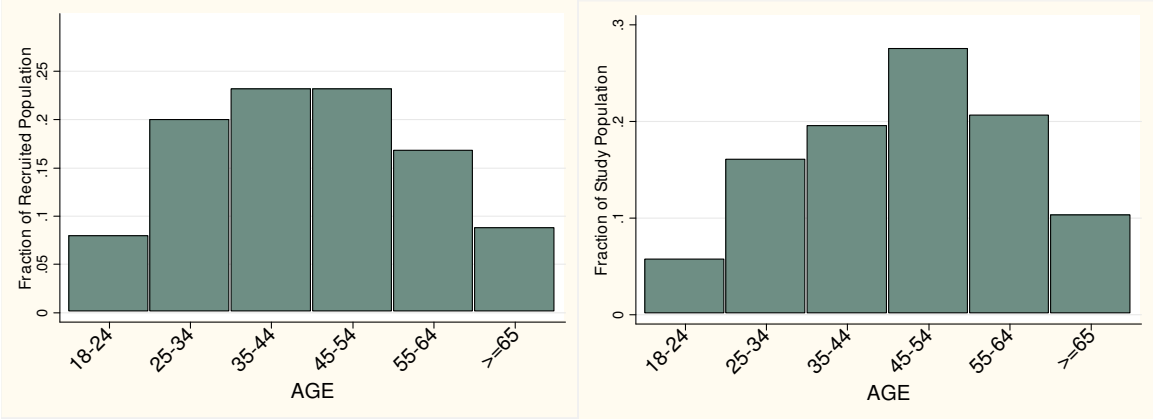
Similar to the overall enrolled population, the demographics of those participants who submitted at least one specimen were well distributed in all categories but race and ethnicity. However, while the overall distributions did not change significantly, there were some notable differences.

As the overall distribution of race/ethnicity was heavily weight toward White/Caucasian the changes in the overall distribution (based on Mann-Whitney test p = 0.2807) did not change significantly. However, upon closer examination of the three race/ethnicity categories (White/Caucasian, Black/African American and Hispanic) there were disparities in those that submitted specimens. 70% (12 people) of Hispanics that were recruited did not submit any specimens. Comparatively, only 26% (25 people) of White/Caucasian enrollees and 18% (2 people) Black/African American participants did not submit any samples. Calculating an odds ratio, recruited Hispanic participants were 10.8 times as likely to not submit any specimens (95% CI 1.35-125.132) than the recruited participants that were White/Caucasian. In other words, there was a statistically higher proportion of Hispanics that were lost to follow up than those who were White/Caucasian (p=0.0068). Comparing the Black/African American recruits to their White/Caucasian counterparts, there was no increase in the odds of submission of specimens (OR = 1.54, [0.29 15.55], p = 0.5934).

The distribution of age in the study population (those that submitted at least one specimen) did not change significantly (Mann-Whitney test p=0.1161) from that of the total recruited population. Age of the actual study population (coded as a categorical variable as mentioned above) still approximates a normal distribution (figure 6.3),

however, compared with that of the total population it is skewed slightly to the right (skew =-0.28 vs. +0.22 of the total population) and more peaked (kurtosis =3.12 vs. 2.3 of total population). In other words, those who actually did submit specimens ("study population") were on average slightly older than the total recruited population. The average age of the study population was 47.3 years old (standard deviation of 13.56) with a median age of 48.1, while the total recruited population had an average age of 44.3 years (standard deviation of 13.9 years) and a median age of 41.9 years

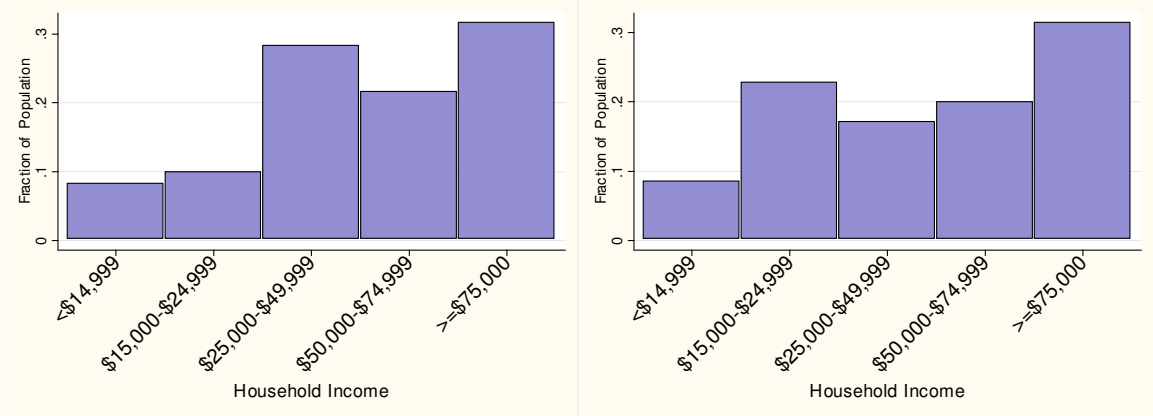
Figure 6.3: Age Distribution of Total Recruited Population (left) Compared with the Study Population (right)



The distribution of gender in the study population also changed slightly. The majority of the total recruited participants were male, representing 54% of the total population. That percentage declined in the study population to 48% of the total participants. Comparing the likelihood of men not submitting samples as compared to women, the odds ratio is 2.14 (95% CI =0.951- 5.14, p =.055), however, the confidence limits include the null value (1.0) and therefore, there is no significant difference in the drop out rate based on gender.

The distributions of income significantly differ when comparing the study population to the total recruited population (Mann-Whitney p = 0.9742). In both groups, the median annual household income range was $50,000 to $60,000. While the overall distributions did not differ, there were some changes. The number of people whose household income was between $15,000 and $25,000 per year decreased from 10% in the total recruited population to less than 5% of the study population. Those whose household income is between $25,00-$50,000 increased from 28% of the total population to 33% of the study population, making this the most prevalent household income category. The other income categories stayed constant relative to the total recruited population (figure 6.4).

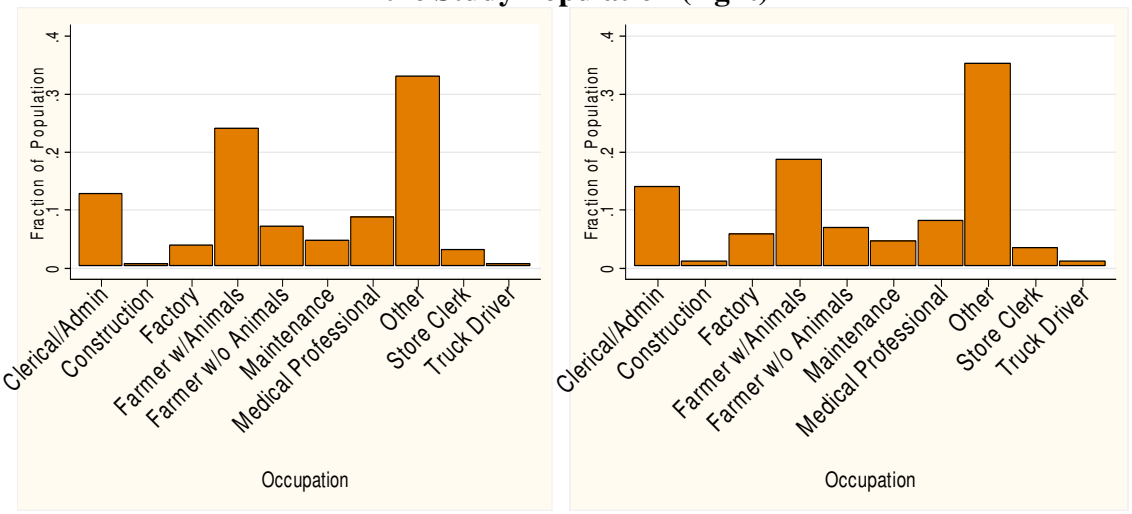
Figure 6.4: Household Income Distribution of the Recruited Population (left) and Study Population (right)



The distribution of study participant occupations between the total people recruited and those actively participating were similar (figure 6.5). However, proportionally, those who were farmers were more likely to not submit specimens than

other occupations (p= 0.0399). When comparing farmers who work with animals and the overall population and row crop farmers with the overall population, it was seen that more animal growers did not participate (p= 0.0287), than row crop farmers (p =0.6006)

Figure 6.5: Distribution of Occupation in Total Recruited Population (left) and in the Study Population (right)



There were only 30 animal growers and 9 row crop farmers enrolled in this study. As farmers are one of the primary groups for comparison in the risk analysis, it was unfortunate that the initial loss to follow up was so high. This resulted in only 15 animal growers and 6 row crop farmers submitting specimens. This initial loss was further compounded among farmers when examining the number of specimens that were submitted throughout the study. Overall, the number of specimens submitted by farmers was low. 35% of the farmers, either associated with row crops or animal agriculture, submitted only one specimen. Of those that submitted more than one, 75% of the farmers (and all but one row crop farmer) submitted four or fewer specimens. Only one row crop farmer submitted more than 4 specimens (7 specimens were submitted) and 5 swine

farmers submitted 7 or more specimens. Only one farmer submitted all 12 specimens. Having such low numbers of specimens, greatly reduced the statistical power of any analyses including farmers as a exposure variable or covariate.

There was a much better response rate of monthly specimen submission by the farm neighbors. Of the 64 farm neighbors, 11% submitted only one specimen, 28% submitted four or fewer specimens and 52% submitted nine or more specimens.

Other Possible Sources of Participant Exposure to Pathogens and Antibiotic Resistant Bacteria

In addition to the demographic factors that may have an impact on the risk of acquisition or carriage of antibiotic resistant bacteria, there are also other possible exposures or factors that may increase or decrease this outcome. For example, if an individual takes certain medications such as antibiotics or antacids, the type and extent of antibiotic resistance of their gut flora can be changed. Obviously, the use of antibiotics provides a direct source of exposure independent of any exposure contribution from animal agriculture facilities affecting the carriage of resistant bacteria in gut flora. Antacids can also have an effect on the gastrointestinal flora, by lowering stomach pH and thereby allowing acid-sensitive bacteria to survive and colonize the intestinal tract. Additional exposure factors for antibiotic resistant bacteria include the source and/or treatment of drinking water, the methods of human wastewater disposal, exposure to natural and man-made water bodies, consumption of under, uncooked or unpasteurized foods, travel outside the country, etc. (data summarized in table 6.2).

Exposure to environmental ground and surface water was of particular importance in this study. Of the study population, 42 people (49%) used environmental surface

waters, such as lakes, rivers/streams, oceans, estuaries etc., regularly for recreational purposes. Of these 42 people, 22 were associated with animal agriculture farms and 20 were associated with row crop farms. Four people had regular occupational contact with environmental waters, all were individuals associated with animal agriculture and three of them were farmers.

Exposure to ground water was determined by the participant's drinking water source. If the individual used well water for drinking, they were considered exposed to ground water. There were fewer study participants exposed to groundwater exposure than exposed to surface water. Of the 87 participants, 25% have private wells as their drinking water source, of which 16 were associated with row crop farms and 6 were associated with animal agriculture. Of the 21 individuals with private wells, only 12 report that the well had been recently tested and only 8 people knew the results of the test. In all 8 of these cases the wells were said to be "ok". The specific water quality parameters for which wells were tested were not available; respondents reported wells were tested for chemicals or they were unsure of what the wells were tested for. None of the 21 wells had been treated with bleach or any other treatment within a year of study enrollment. Of those that do not have private wells as a water source, three people are unsure of the source of their water, and all others have community or county water systems as their source of drinking water.

Wastewater disposal in the study communities is predominantly septic systems, With 97% of study households reporting septic tanks as opposed to city or county sewer systems.

Because a common source of exposure to antibiotic resistant or pathogenic bacteria is under-cooked or uncooked food, each person was asked about their consumption of raw or rare meat and unpasteurized soft cheeses. Eight (8) people (2 associated with row crop farms/6 with animal agriculture) reported eating rare or raw meat and 9 people (5 associated with row crop/ 4 with animal agriculture) reported eating soft cheese. Of the 9 people reporting consumption of soft cheese, there was some question as to whether or not the cheese was unpasteurized. The question was asked "Do you eat soft Mexican cheese or other unpasteurized products?" It is possible that people interpreted soft Mexican cheese as "queso dip", which often found at Mexican restaurants and is usually a pasteurized product.

Pets are another source of exposure to antibiotic-resistant or pathogenic bacteria. Eighty (80) percent of study participants (68 people) reported having at least one pet, with 67% having dogs, 37% having cats, 6% having birds and 18% having some "other pet." Numbers of people having "other" animals included: 3 having rodents such as rabbits or hamsters, 4 fish, 6 horses, 4 goats or sheep and 3 poultry birds, including a rooster and peacocks. None of the pet owners reported giving their pets any medications to treat illness.

Exposure to other people who may harbor pathogenic or resistant bacteria is another potential source of risk. Such exposure risks can occur in hospital/doctors offices or during foreign travel. Foreign travel (travel abroad) was reported by 30% of people, however, only 8% (7 people) had traveled within one year of enrolling in the study. Of these 7 reporting travel within 1 year of enrollment, 2 traveled to the Bahamas, 1 to Cozemel, 1 to Canada, 1 to England and 1 to Germany. No one reported being ill during

their travels. One person reported taking medications during their travels but this trip was in 2004 and not within one year of study enrollment.

Four people who were born in Mexico and moved to the United States as adults, of which two had been in the United States at least 5 years prior to enrollment in the study, one had arrived as recently as March 2005 and one did not report the date of arrival arrived in the United States

Twenty (20) percent of the study population (17 people) reported being treated by a doctor within 30 days of enrolling in the study. One of these people went to the doctor for treatment of a diarrheal disease while all others (16 people) went for routine exams or check ups. In addition to those who visited doctors, 4 people reported having been admitted to the hospital within the six months prior to enrollment in the study. One was treated for pneumonia, one for intestinal or stomach problems, and the other two for back problems and anxiety, respectively.

Most people reported feeling well on the day of the interview. However, one person was ill with a diarrheal ailment. Additionally, six (6) people reported having diarrhea within the two weeks prior to the enrollment interview. Some study participants had a history of chronic illness. Three (3) people reported having diabetes, 4 reported stomach or intestinal problems, two reported lung problems and two reported having had cancer/leukemia. Though not on the list of chronic illness in the questionnaire, one person reported having Parkinson's disease and was included in the category for chronic illness.

Many of the study participants reported taking various types of medication. These drugs ranged from over the counter medications such as pain and allergy

medications, to prescription drugs for a variety of diagnoses including high blood pressure, high cholesterol, arthritis, heart disease etc. Medications that can have an effect on the bacteria in the gastrointestinal tract include antibiotics, antacids and steroids. Six (6) people reported having taken antibiotics within six weeks of enrollment in the study, of which 2 were associated with row crop farms and 4 were associated with animal agriculture. Five (5) people reported taking some type of steroid within six months, of which 2 were associated with row crop and 3 were associated with animal agriculture. Nineteen (19) people (22%) reported regularly taking antacids, of which 8 were associated with row crop farms and 11 were associated with animal agriculture.

An additional 18 people reported having taken antibiotics during one or more months of submitting fecal specimens. In most cases, antibiotic usage was to treat an acute illness, or as a preventative for surgery. In one case the antibiotics were taken for acne treatment. Of the total of 24 people who took antibiotics prior to or during the course of the study, 13 are associated with row crop farms and 11 are associated with animal agriculture. One additional person began taking antacids regularly during the study, giving a total of 20 people continuously taking antacids.

Table 6. 2: Summary of Number and Percentage of People to Potential Exposures to Antibiotic Resistant Bacteria and Enteric Pathogens

Exposure	# of people	% of total	# associated with CAFOs (%)	# associated with Row Crop Farms (%)
Recreational Surface Water	42	49%	22 (49%)†	20 (50%)
Ground Water	21	25%	6 (13%)	16 (80%)
Septic Systems	84	97%	44 (94%)	39 (100%)†
Soft Cheese	9	10%	4 (9%)	5 (13%)
Rare/Raw Meat	8	9%	6 (13%)	2 (5%)
Pets	68	80%	33 (73%)†	35 (88%)
Foreign Travel	7	8%	4 (9%)	3 (8%)
Foreign Born	4	5%	4 (8.5%)	0
Doctor Visit	17	20%	9 (19%)	8 (21%)
Hospital Stay	4	5%	3 (6%)	1 (3%)†
Chronic Disease	12	14%	6 (13%)†	5 (13%)†
Use of Steroids	5	6%	3 (7%)†	2 (5%)
Use of Antibiotics	6[+18]*	28%	15 (32%)	13 (33%)
Use of Antacids	19[+1]*	22%	12 (26%)	8 (20%)

* The initial number is those who reported use in initial questionnaire. The "+#" is the number who report usage in monthly questionnaires

† The percentage is based on the total number of people who answered the question. There were several instances in which one or two people did not know or did not respond

Active Study Population Compared by Exposure Group

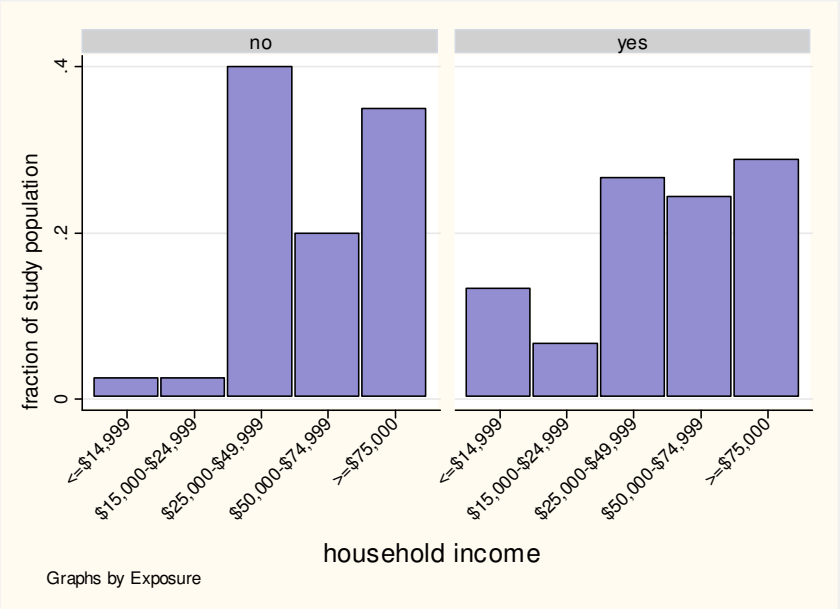
Of the 87 active participants, 47 (54%) are employees or neighbors of an animal agriculture facility, and 40 (46%) are employees or neighbors of a row crop farm. As this study is a prospective cohort study, the participants were not matched based upon demographic characteristics. However, for comparison purposes it is important to characterize similarities or differences between these 2 groups.

Race/ethnicity was the only variable for which there was a major difference between the exposure groups. The proportion of minorities in the row crop communities was significantly lower than the proportion in CAFO communities (p = 0015). Of those

associated with the row crop farms, 98% of the participants are Caucasian/White, one was African American/Black and none were Hispanic. In the group associated with animal agriculture, 72% were Caucasian/White, 17% (8 people) were African American/Black and 11% (5 people) were Hispanic.

There were small differences in the exposure groups with regard to income (figure 6.6), though the overall distributions are not significantly different in these two groups ($p = 0.092$). In general, more people associated with animal agriculture facilities had a household income of less than $25, 000 than those associated with row crop facilities, specifically 9 people (19%) associated with animal agriculture and 2 people (5%) associated with row crop farms. Examining the other three income categories: in the $25,000 to $50,000 range, there were 16 people associated with row crops compared with 12 people associated with animal agriculture; in the $50,000 to $75,000 range there were 8 people associated with row crop farms compared with 11 associated with animal agriculture; and of those with a total household income of $75,000 or more, the two exposure groups were about the same, with 14 people associated with row crop farms and 13 associated with animal agriculture.

Figure 6.6: Income Distribution in Study Population by Exposure

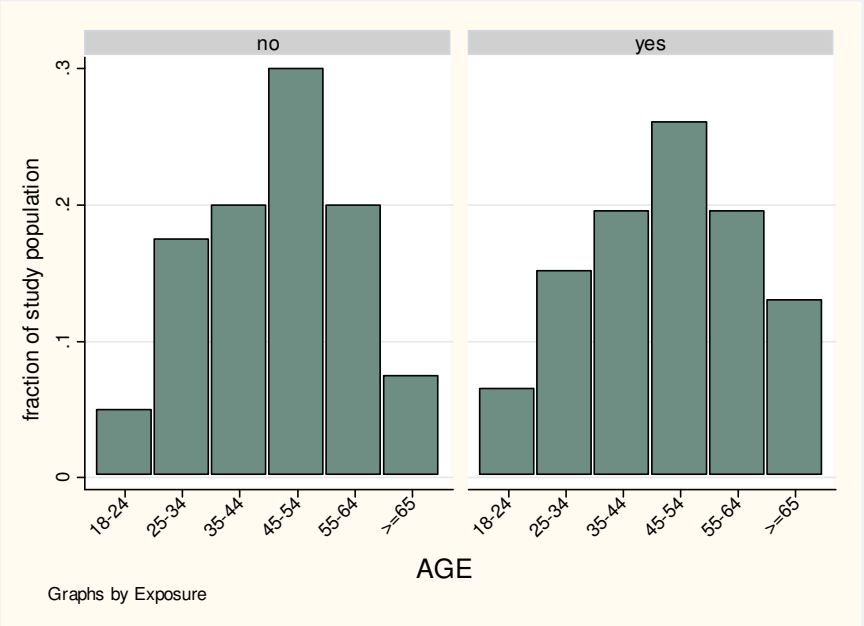


For gender the distribution between the two study groups were almost even. In CAFO communities, 55% of the participants were women and 45% were men and 48% women and in Row crop communities, 48% of the participants were women and 52% were men. Comparing the proportions of men to women in the two communities, there was no significant difference in the two groups (p =0.4670).

Age distributions were also similar between the two exposure groups and both distributions approximate normality (p >0.10 for normality test in each distribution) (figure 6.7). The average age per exposure group was similar at 47.6 and 46.9 years of age in CAFO communities and row crop communities, respectively. The distributions of age in the two communities are not significantly different (p = 0.817), however, the distribution among the unexposed (row crop farm) group is more peaked than the exposed group. Almost one third of the unexposed group is in 45-54 category compared with only one fourth of the exposed group in this category, the unexposed group has

fewer people older than 65 years of age, and all other age categories had approximately equal number of people.

Figure 6.7: Comparison of Age Distribution by Exposure Group



Fecal Specimens Submitted and Pathogenic and Antibiotic-resistant Bacteria Isolation

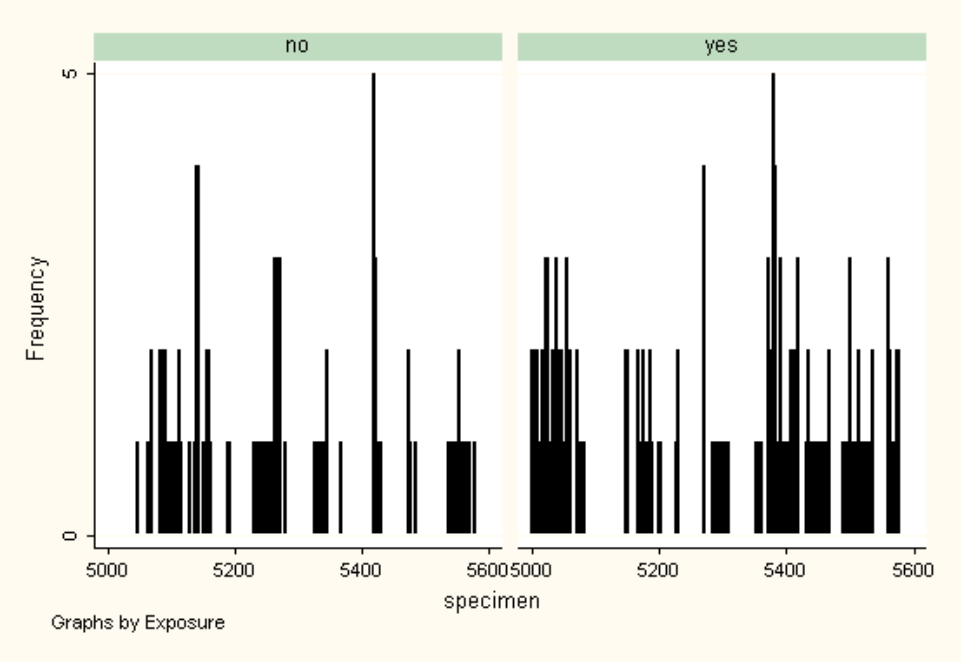
A total of 578 specimens were received at WFUBMC. All specimens received were regular monthly submissions; there was only one instance of specimen submission due to diarrheal illness. No *Salmonella* or *Campylobacter* were isolated from any of the specimens. *Salmonella* were analyzed by direct plating the specimen onto Hektoen agar. This is a specialized agar for *Salmonella*, *Shigella* speciation. *Campylobacter* was analyzed for by direct plating a portion of the specimen onto Blood Agar and incubated in a CampyPak Plus microaerophillic system.

Of the 578 specimens, 285 (49%) did not yield *E. coli* or *Enterococcus* sp isolated on specialized agar (MacConkey Agar and Enterococcasil Agar, respectively) impregnated with low level antibiotics: including tetracycline (4 μg/ml), ciprofloxacin (2 μg/ml), norfloxacin (4 μg/ml) and gentamicin (4μg/ml) for the *E. coli* and tetracycline (4 μg/ml), vancomycin (8 μg/ml), ampicillin(8μg/ml), gentamicin (250μg/ml), streptomycin (250μg/ml), and Quinupristin/Dalfopristin (2μg/ml (Table 6.1).

Of the remaining fecal specimens, 106 had at least one *E. coli* that was resistant to one of the four prescreening antibiotics and 226 specimens had at least one Enterococcus isolate with resistance to one of the six prescreening drugs (table 6.1). Some specimens yielded both *E. coli* and Enterococci.

The number of resistant isolates per specimen ranged from 1 to 5 *E. coli*, *Enterococcus* or both. The majority of specimens only had one only isolate (figure 6.8). There were a total of 154 positive specimens submitted by people associated with CAFOs and 143 positive specimens submitted by people associated with row crop farms. Specimens with multiple isolates were submitted by people in both groups.

Figure 6.8: Number of Isolates per Specimen Submitted in those Associated with Row Crop Farms (left) and CAFOs (right)



Of the 87 people who submitted specimens, only 16 people did not have antibiotic resistant targeted enteric bacteria detectable in any of their stool samples. Twelve (12) of these individuals were associated with row crop farms and 4 were associated with swine farms. There were 13 people for which all of their specimens had at least minimally resistant targeted enteric bacteria present. For all other participants, more than one of the specimens had resistant bacteria and at least one specimen did not contain any bacteria with resistance. Because each person submitted different numbers of specimens and at different times, it is difficult to compare the frequencies and temporal patterns of positive and negative fecal specimens based on specimen submissions alone. There are some people for which all submitted specimens were negative or all were positive for resistant

bacteria while other people had positive specimens followed by negative specimens and then positive specimens again.

The 87 study participants who submitted fecal specimens and the resulting information on presence of antibiotic-resistant *E. coli* and Enterococcus bacteria in these samples were used as the basis for a risk analyses. If one or more of the submitted fecal samples was positive of antibiotic-resistant target bacteria, as described above, the participant was considered to be positive for the outcome – carriage of antibiotic resistant bacteria.

Risk Analysis

The following analyses are based upon the presence or absence of antibiotic-resistant target bacteria harbored by the 87 people in the study population described above who submitted fecal specimens. It is important to note that these analyses are based upon carriage of antibiotic resistant enteric bacteria and not upon the specific sources of antibiotic bacteria from which acquisition of resistant bacteria could have occurred, such as from animal agriculture facilities. While the people who live near or work on animal agriculture facilities are denoted as the "exposed" group and those associated with non-animal agriculture facilities are the "non-exposed" group, a specific link between the bacteria found in the humans and those found in the environmental samples has not been established based on either phenotypic or genetic properties of these bacteria. The phenotypic antibiotic resistance profiles of the bacteria isolated from people, from swine wastes of agricultural facilities and from ambient surfaces waters of participating have been characterized. However, this information on the occurrence and

properties of these bacteria was adequate to determine the source of the bacteria found in the human stool samples of the study participants.

The incidence of carriage of antibiotic resistant bacteria in the overall study population was high. Almost 82% of this population had at least one specimen with resistant *E. coli* or *Enterococci* sp. As the duration of the study was different, due to significant loss to follow up, "incidence" refers to at least one specimen with resistant bacteria over the course of the year. However, it is not standardized based upon loss to follow up. In other words, incidence is considered per person year regardless of the number of months a person is actively participating. Potential bias in this approach is acknowledged, however, as this is a pilot study and there was significant loss to follow-up, this is believed to be the most informative approach.

To assess risk, it is necessary to compare the incidence proportion of carriage in the two exposure groups. Essentially this ratio is:

$$(A_1/N_1)/(A_0/N_0), \qquad \textbf{eqn 6-1}$$

where A_1 is the number of people with resistant bacteria in the exposed group, N_1 is the total number of people in the exposed group, A_0 are those with the outcome in the non-exposed group and N_0 is the total number of people in the non-exposed group. While this comparison seems mathematically trivial, the comparison gets more complicated as other variables are considered. Therefore log-linear regression is used to estimate the risk ratio. A Log-linear model is able to directly estimate the Risk Ratio parameter. In instances in which the outcome is not rare (as in this study) log-linear regression is the most appropriate model to use to assess risk between two populations.

The crude log-linear model used is that in which

Probability of Outcome = prediction variable = $\alpha + \beta(X)$ **eqn 6-2**

where outcome is carriage of AR bacteria and the prediction variable is association with animal agriculture. This model compares the incidence proportion of the exposed group to that of the unexposed group. This model becomes more complex as other variables are included to assess there potential impact on the result. The model then becomes:

$\text{Prob(Outcome)} = \alpha + \beta_1(X_1) + \beta_2(X_2) + \ldots \beta_n(X_n)$ **eqn 6-3**

Where β_1 is the regression coefficient for the exposure variable and $\beta_{2\ldots n}$ are the regression coefficients for other variables that may be included in the model such as age, gender, race/ethnicity etc.

When assessing the basic (or crude) model (eqn 6-2) of the effect of exposure (living near or working on swine farms) on carriage of antibiotic resistant bacteria an effect is seen. The risk ratio is 1.31 (CI =1.05, 1.63) with a Prob>|z| = 0.017. As the confidence intervals do not cross the null value (null =1), and the p value is less than the α of 0.05, this effect is considered statistically significant. This estimate implies that those who live near or work on animal agriculture facilities are 0.3 times (30%) more likely to carry antibiotic-resistant bacteria in there gastrointestinal bacterial flora than those who live near or work on row crop farms.

As indicated above, there are many factors that have the potential to affect a person's carriage of antibiotic resistant bacteria. Some of these variables include gender, race, amount of contact with animals, use of various medications, diet, travel, exposure to environmental water, visits to hospitals, clinics or doctors' offices and overall health of the individual. Many of these variables were characterized in the initial enrollment

questionnaire and additional information was provided in the monthly questionnaires that accompanied the submitted fecal specimens. To understand potential impacts of each of these variables, risk estimates were calculated based on each of the variables being the exposure rather than the farm type with which the individual is associated. The resulting model is:

$$\textbf{Outcome} = \alpha + \beta_1(\textbf{assessed variable}) \qquad \textbf{eqn 6-4}$$

For this analysis each variable is coded as a dichotomous variable for exposure. This includes the age variable which has previously been coded as a categorical variable based on 10 year increments. The dichotomous age variable was established using the median value of age (48 years) as the cut off. Those who are 48 years old or younger are considered unexposed while those over 48 are considered exposed. Further analyses use the categorical age variable, the dichotomous age variable or both. The variable used is indicated.

The risk ratios established in these models (table 6.3) help describe the data and allow for better understanding of their impacts on the final model. Furthermore, they can give further insight into which variables may be potential confounders. There are two variables for which a statistically significant effect is seen when variables are assessed as the exposure. These are farm association (RR= 1.31 (1.05- 1.63)), which is the actual exposure being analyzed in this study, and hospital stay (RR = 1.23 (1.11-1.37)). For all other variables the 95% confidence intervals included the null value of 1 (table 6.3).

Table 6.3: Impact of potential covariates on outcome when assessed as the exposure variable

Assessed variable	Coding	Risk Ratio	95% Confidence Interval
Farm association	0 = row crop 1=animal agric	1.31	1.05-1.63
Farmer	0 = non-farmer 1= farmer	0.81	0.61-1.08
Race/ethnicity	0 = White 1= any other race/eth	1.06	0.83-1.35
Gender	0 = female 1= male	0.98	0.81-1.2
Antibiotic use	0=did not use 1=used at least once	1.02	0.82-1.27
Antacid use	0=did not use 1=used at least once	1.11	0.92-1.34
Pets	0= do not have 1= have	1	0.78-1.28
Steroids	0=did not use 1=used at least once	1.25	1.12-1.39
Hospital stay	0=no stay 1=hospital at least 1 day	1.23	1.11-1.37
Foreign travel	0 = no 1= yes	0.86	0.66-1.11
Drinking water source	0 = city/county water 1= well	0.85	0.65-1.12
Use Environ water for Recreation	0 = do not use 1= use	0.91	0.75-1.11
Eat soft Cheese	0= do not eat 1 = does eat	0.66	0.36-1.19
Eat Raw/rare meat	0= do not eat 1 = does eat	0.91	0.60-1.38
Doctor Visit	0= no visits 1= at least one visit	1	0.78-1.28
US native	0= yes 1= no	0.92	0.52-1.63
Chronic Illness	0= no illness 1 = yes illness	1.01	0.75-1.37
Age (as a dichotomous variable)	0 = <=median age (48) 1= >median age	1.19	0.97-1.46

In addition to analyzing each potential covariate alone as the exposure variable, each variable was assessed as a covariate based upon its interaction with the actual study exposure (farm association). This was done to determine the potential of each variable as a confounder of the risk estimate. An adjusted log-linear model to determine a risk ratio for each variable along with the outcome and exposure was used (eqn 6- 4).

$$\textbf{Outcome} = \alpha + \beta_1(\textbf{exposure}) + \beta_2(\textbf{assessed variable}) \qquad \textbf{eqn 6- 5}$$

To determine the potential for confounding, the risk ratios of the adjusted models (table 6.3) were compared with the crude model (exposure and outcome alone) and the impact of any given variable is determined by a ten percent change in the natural log of the risk estimates. In other words, if the overall natural log of the risk estimate changes by 10% or greater when the variable is included in the model, it is said to have an impact on the probability of the outcome and is therefore maintained in the model for the final risk estimate. The percent change is calculated using the following equation:

%change =(ln(modified)-ln(crudeRR))/ln(crudeRR) **eqn 6-6**

There were six variables for which there was at least a 10% change in estimate (table 6.4). These variables include: age (categorical), antibiotic usage, chronic illness, having pets, the source of drinking water and eating soft cheese. Due to the uncertainty of true meaning of responses with regard to consumption of soft cheese as a pathogen or antibiotic-resistant bacteria risk factor as mentioned earlier, it was not be included in the larger model even though a greater than 10% change estimate was observed. Hospital stay was determine above to have an impact on the outcome when assessed as the exposure variable, however, when assessing its impact on the model with farm association as the exposure the model would not converge. This could be due to the fact that there were only seven people who stayed in the hospital for at least one night within six months of beginning the study or during the study period. With such a low number of people, there were zero values in the equation that would not allow the model to converge. Further analyses were also deemed to be not necessary as all but one of the hospitalizations resulted from an illness that required antibiotics. As antibiotics use is included in the model, accounting for the hospital stay as a potential exposure to

antibiotics would be redundant. Therefore, using this variable as a covariate would introduce confounding rather than reduce it.

Table 6.4 :Risk Ratio Estimates, 95% Confidence Limits, p-value and % Change in Estimates for Models including Different Variables that may Impact the Risk Estimation of Carriage of Resistant Bacteria

Model Variables	Risk Ratio	95% Confidence Interval (precision)*		P-value	% change
exposure (crude model)	1.31	1.05- 1.63	(1.55)	0.017	--
exposure + farmer	1.28	1.04-1.58	(1.77)	0.020	-7.5%
exposure + age (categorical)	1.25	1.06-1.48	(1.40)	0.008	-15.9%
Exposure + age (dichotomous	1.28	1.03 -1.58	(1.53)	0.026	-8.9%
exposure +gender	1.32	1.06 -1.65	(1.56)	0.013	4.3%
exposure + race	1.31	1.05-1.64	(1.56)	0.019	0.6%
exposure + doctor visit	1.29	1.03-1.61	(1.56)	0.025	-5.3%
exposure + antibiotic usage	1.27	1.02-1.58	(1.55)	0.034	-11.0%
exposure + antacid use	1.35	1.09 -1.67	(1.53)	0.06	3.0%
exposure +steroid use	1.29	1.04-1.60	(1.54)	0.021	-5.1%
exposure +chronic illness	1.34	1.07-1.68	(1.57)	0.011	10.0%
exposure + pets	1.35	1.08-1.69	(1.56)	0.007	13.2%
exposure +foreign travel	1.28	1.02-1.60	(1.57)	0.030	-7.2%
Exposure + immigration	1.33	1.06-1.65	(1.56)	0.012	5.5%
exposure + water source	1.39	1.12-1.71	(1.53)	0.002	21.9%
exposure +recreational water use	1.27	1.03-1.58	(1.53)	0.026	-9.6%
Exposure + eat soft cheese	1.27	1.03-1.57	(1.52)	0.027	-10.9%
Exposure +eat raw/rare meat	1.31	1.06-1.64	(1.55)	0.014	2.3%

* Precision is the confidence interval width (upper limit / lower limit)

Considering all the identified study variables, a 10% change is considered to be the cut-off value, and given the removal of hospital stay and soft cheese consumption, there were five covariates added to the model. The adjusted model becomes:

$$\textbf{Prob(outcome)} = \alpha + \beta_1\textbf{(exposure)} + \beta_2\textbf{(drinking water source)} + \beta_3\textbf{(chronic illness)} + \beta_4\textbf{(pets)} + \beta_5\textbf{(antibiotic use)} + \beta_6 - \beta_{10}\textbf{(age category)} \quad \textbf{eqn 6-7}$$

The age variable is categorical and therefore, dummy variables must be used to account for each age category. As a result, there are five β coefficients in the model; one for each dummy variable.

The risk estimate for this model was assessed as well as interaction of the different covariates. A backwards elimination approach was then used to determine if all variables are in fact required or if one or more variables confound one another. This technique begins with the full model and removes one variable at a time. As done above in comparing the crude to the adjusted models, the percent change in estimate was assessed as each variable was removed from the model. In this case however the full model is compared with that of the removed covariate.

With some of the larger models there were problems with convergence. As seen with the adjusted model including hospital stay, there are instances in which the added variables generate stratification that results in too few cell counts for the models to converge. This problem is further compounded by missing data. In some cases there are one or more people that did not respond to a given question. As the model gets larger the potential for missing date multiplies which may result in an even smaller study population.

The convergence problem arises with age (as a categorical variable) and with antibiotic use. If antibiotic use is removed from the model and dichotomous age is used as a surrogate for categorical age, then the model will converge with four of the five covariates in the full model. Doing so results in a risk ratio estimate of 1.37 (1.11-1.69). As taking antibiotics is very likely an additional source of exposure however, it is important to keep it in the model. Antibiotic use can be included in a model with as many as three covariates and the model will converge. Adjusting for antibiotic use, chronic illness and drinking water source, the risk ratio is 1. 37 (1.09-1.71) and adjusting for antibiotic use, drinking water and pets the risk ratio is 1.42 (1.17-1.72). Using a backward elimination from these two models with three covariate, and assessing antibiotic use and drinking water as the covariates, the risk ratio estimate is 1.34 (1.08 – 1.69). Comparing this with the model including antibiotic use, drinking water and having pets (RR=1.42), there is a 10% change in estimate and therefore, all three variable should remain in the model. When comparing to the three covariate model with antibiotic use, drinking water source and chronic illness (RR= 1.37), there is only a 7.6% change which indicates that chronic illness may be removed from the model. When removing drinking water from the model and therefore comparing the three covariate model to one which includes only antibiotic use and having pets (RR = 1.34), there is a greater than 10% change as well. Therefore it is concluded that these three covariates, antibiotic use, drinking water and having pets should be included in the final model. This results in a the following final model:

$$\textbf{Prob(outcome)} = \alpha + \beta_1\textbf{(exposure)} + \beta_2\textbf{(drinking water source)} + \beta_3\textbf{(antibiotic use)} + \beta_4\textbf{(pets)} \qquad \textbf{eqn 6-7}$$

Considering all the factors the final model estimates a Risk Ratio of 1.42 (1.17-1.72). This estimate is considered to be statistically significant as the confidence interval does not include the null value of one. The precision of this estimate as determine by the confidence interval width (Upper limit/Lower limit) is 1.47. Given the small sample population, this width is relatively narrow and therefore, relatively precise. Furthermore, adjusting for the covariates did make the estimate more precise than the crude model (precision = 1.55) or adjusting for any of the three covariates alone in an adjusted model (for the three variables the precision was greater than 1.60 in the adjusted models).

Risk Associated with being a farmer

While accounting for being a farmer or not did not have an impact on the overall risk analysis, there is interest in understanding the potential risk of working in animal agriculture as opposed to working in a non-animal agriculture setting. In this study there were 23 people who submitted at least one specimen that are farmers. As mentioned above, 9 of those are row crop farmers and 14 are animal agriculture growers. When comparing these two groups, utilizing log-linear regression, the Risk Ratio is 1.93 (0.90 – 4.13). While there appears to be a larger effect for those who work with the animals as opposed to those who work on or are neighbors of animal facilities (RR=1.31), the confidence interval includes the null value and therefore the result is not statistically significant. It is important to note however, that the precision of this estimate is relatively large (4.59 compared with 1.55). These wide confidence limits can be attributed to the small number of farmers in the study. With a larger study population,

the confidence interval would have been narrower thus resulting in a more precise estimate.

While the results of this study do not allow us to conclude that there is a higher risk of antibiotic carriage associated with working with live stock, there are other studies that have quantified an increased risk to those who work with animals compared with those who do not. Levy et al. (1978) found that families that worked or lived on poultry farms in the United States had a higher incidence of carriage of antibiotic resistant bacteria than those who lived in town. In addition, a study in France demonstrated that those working in swine facilities had a higher incidence of antibiotic resistance carriage than non farmers living in the same areas as the farms (Aubry-Damon et al., 2004).

Summary

The original research question concerning the risk of acquiring antibiotic resistant bacteria originating from animal agriculture facilities was not definitively answered in this study. This is because concentrations and antibiotic resistance profiles of the bacteria found in the environmental waters and human stool samples were not conclusively linked to the animal agriculture facilities or row crop farms studied. There were several possibilities why this occurred (Chapter 5), including high background levels of bacteria in the water that may have masked contributions of the farms, and relying solely on phenotypic analyses to assess links of origin in the bacterial isolates.

Even without these conclusive links however, the results of this study yielded valuable data on antibiotic-resistant bacteria presence on animal agriculture and row crop farms and in people working on and living near these farms that provide a much better

understanding of antibiotic resistant bacteria human carriage and occurrence swine waste and environmental waters of rural communities in eastern North Carolina. Furthermore, it was possible to analyze the two exposure groups, people geographically associated with swine agriculture farms and with row crop farms with regard to carriage of antibiotic resistant bacteria. The risk of antibiotic-resistant bacteria carriage was significantly higher in people associated with swine agriculture farms compared to those associated with row crop farms (unadjusted RR = 1.31, 95% CI = 1.05 to 1.63). However the sources of the antibiotic-resistant bacteria harbored by the participants of this study are unclear based on the study findings.

This study found that there was a very high rate of carriage of antibiotic resistant bacteria within the study population as a whole. Furthermore, while not all racial and ethnic groups were adequately represented, based upon demographics of the entire regional population, in this study, many other demographic factors such as age, race, income and gender were well represented in this research.

Comparing the two study populations, there was a higher occurrence of antibiotic resistant bacteria carriage among those people who live near or worked on animal agriculture facilities when compared to those who are associated with non-animal agriculture (row crop) facilities. This relationship held true in the crude model in which only exposure status and outcome was assessed, as well as in models for which other potentially confounding variables, including source of drinking water, medication usage, having pets and overall health of the participant, were included.

After assessing all the potential cofounders and relevant demographic variables, it was found that a model including the outcome, type of farm association (exposure), the

source of drinking water, having pets and use of antibiotics during the study was the most appropriate. Using this model, there is a risk ratio estimate of 1.42 (95% CI = 1.17 - 1.72). As the confidence limits do not include the null value, this estimate is considered to be statistically significant. Given this estimate, it is concluded that there is an increased risk of antibiotic resistant bacteria carriage in those who are associated with animal agriculture when compared with those associated with non- animal agriculture. In other words, people who live near or work on animal agriculture facilities are 1.4 times more likely to carry antibiotic resistant bacteria in their gastrointestinal tract than those who live near or work on row crop farms.

Chapter 7 – Discussion and Conclusions

Discussion

Antibiotic resistant bacteria and zoonotic pathogens are of concern regarding their impact on public health. Bacteria pathogens such as *Salmonella*, *Campylobacter* and enterohaemorrhagic strains of *E. coli* have been associated with food animals including swine, cattle and poultry (Aarestrup, F.M. and Wegener, H.C., 1999, Smith, K.E. et al., 1999, Molbak, K. et al., 1999, Schroeder, C.M. et al 2002, WHO, 2004 and Gebreyes, W.A. 2005) and studies have linked the consumption of these animal products to human illness. In addition to the frank pathogens, antibiotic resistant bacteria have also been linked to animal agriculture facilities and animal products (Aarestrup F.M. and Wegener, H.C. 1999, Schroeder, C.M. et al 2002, Kuhn et al., 2005, Manero et al., 2006, Messi, P. et al 2006, Stine, O.C., 2007). Antibiotic resistance has been documented in many different bacterial species including human pathogens and commensal organisms of these food animals. While many of these bacterial species found in animals do not permanently colonize the human gastrointestinal (GI) tract, it has been demonstrated that they can survive in the GI tract for several days to weeks (Aarestrup, F.M. and Wegener, H.C., 1999, Sorensen et al., 2001). This survival allows interaction between these environmental bacteria present in the GI tract and the human bacteria also located there. This interaction can then lead to the transfer of resistance traits to human bacteria

including pathogens; this in turn may lead to the emergence of antibiotic resistant pathogens for which there are no treatments.

Previous research has documented that consumption of meat can lead to ingestion of antibiotic resistant bacteria from food animals (Aarestrup F.M. and Wegener, H.C. 1999, Sorensen T.L. et al., 2001, Buscani, L. et al., 2004, Jackson, C.R. et al 2007). However, exposure to resistant bacteria by other routes is less understood. A study by Johnston, L.M. and Jaykus, L. (2004) found that produce fields fertilized with or sprayed with untreated animal waste can result in the contamination of the produce with antibiotic resistant bacteria. This provided evidence of one potential route of environmental exposure for antibiotic resistance from animal agriculture, specifically produce.

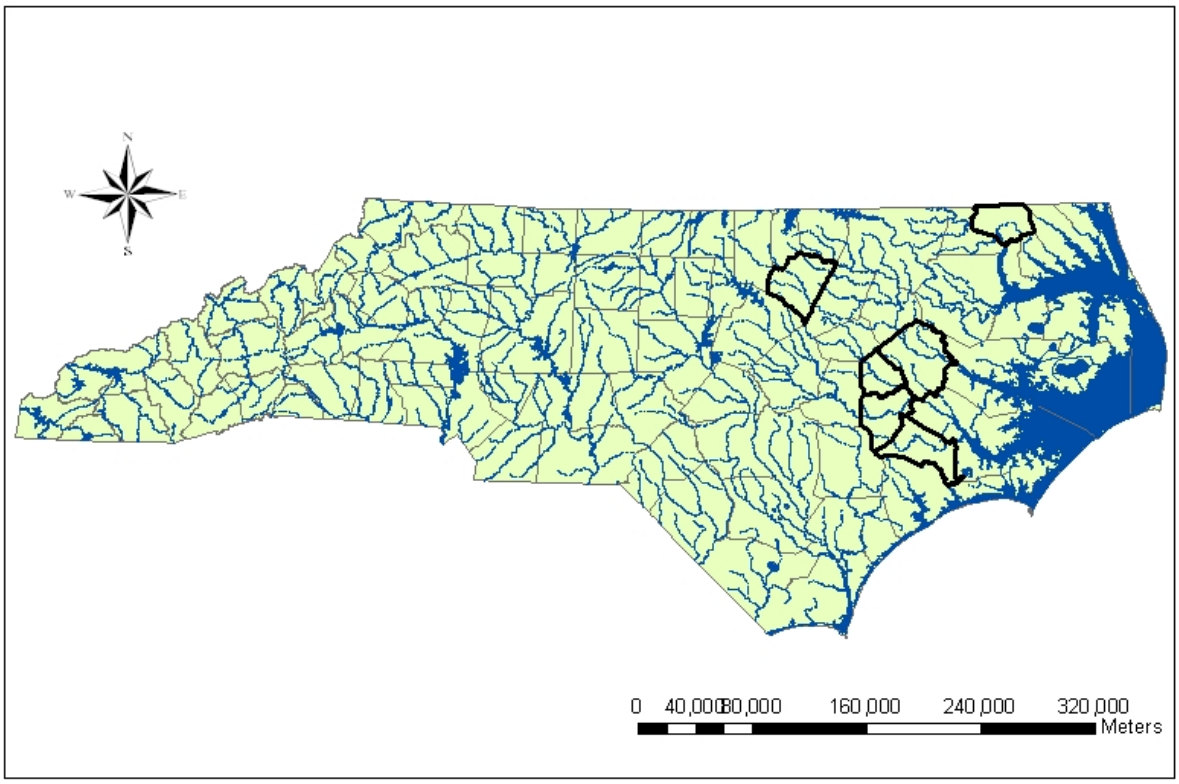
Our study was intended to examine the risks of people acquiring antibiotic resistance bacteria from animal agriculture via exposure to potentially contaminated ground and surface water that flows through or adjacent to animal agriculture facilities. Furthermore, the study was designed to elucidate the incidence of carriage of antibiotic resistant bacteria in rural populations, specifically those that live near or work on various types of farms (CAFOs and row crop farms).

This was a pilot study conducted to better document and understand the potential risks of antibiotic resistant bacteria and enteric pathogens originating in animal agriculture to people who live near or work on animal agriculture farms. While there were some obstacles in conducting this research, the overall research goals and objectives were basically achieved.

Environmental Sampling

In this study 11 swine farms (10 of which also grazed cattle) were analyzed and compared with 6 row crop farms to determine the effect if any, of animal agriculture on the concentrations of antibiotic resistant, enteric bacteria in ground and surface waters surrounding the farms. All of the study farms were located in eastern North Carolina counties (figure 7.1). For all farms, up and downstream surface water samples were collected; and on the animal agriculture facilities ground water and animal waste samples were also collected. All the samples were analyzed for three enteric bacteria including the frank pathogen *Salmonella* and commensal bacteria *E. coli* and Enterococci. The bacteria from each sample were quantified, biochemically identified and characterized for phenotypic antibiotic resistance traits. Up and downstream water samples were then compared to determine the impact of the farm itself on the environmental water. Water samples associated with animal agriculture were then compared with those associated with the row crop farms to determine if there were any differences.

Figure 7.1: Counties in which Study Farms are located



In addition to the environmental sampling, people who lived near or worked on any of the study facilities were asked to submit fecal samples once per month for one year. Each sample was analyzed for the same enteric bacteria as in the environmental samples. The bacteria isolated were then characterized for antibiotic resistance traits and each individual was scored positive or negative for antibiotic resistance carriage. Furthermore, the antibiotic resistance profiles of the bacteria found in the people were then compared to those of the environmental bacteria to determine any similarities that may provide information regarding the source of the bacteria found in the human participants.

In the environmental analyses, animal waste samples had the highest concentrations of bacteria in all of the environmental samples. Concentrations of *E. coli*

and Enterococcus were as high as 8.2 $\log_{10}$cfu/100ml in swine waste (barn flush samples) and cattle manure. The concentrations in lagoon samples were statistically lower ($p<0.0001$) than the barn flush samples, but were still relatively high (geometric mean =4.7$\log_{10}$cfu/100ml). As would be expected the concentrations of *Salmonella* were much lower than those of the indicator organisms, *E. coli* and *Enterococcus* sp., in all samples; the geometric mean concentration of *Salmonella* was 2.3$\log_{10}$cfu/100ml in barn flush and 1.4$\log_{10}$cfu/100ml in lagoons. *Salmonella* sp. were found in some cattle manure samples as well, however, concentrations were much lower in the cattle manure (-0.8 $\log_{10}$cfu/g) than in the swine waste.

In the surface water samples, the indicator bacteria were always detected. The concentrations of *E. coli* (geometric mean *E. coli* = 2. 3$\log_{10}$cfu/100ml) were similar regardless of where the sample was taken (up or downstream of the farm) or with which farm type the stream was associated. This was also the case for *Enterococcus* concentrations (geometric mean *Enterococcus* = 2.2$\log_{10}$cfu/100ml). There was no statistical difference in the concentrations of bacteria that were isolated up or downstream of row crop farms, or up or downstream of animal agriculture facilities.

The generally high concentration of *E. coli* and *Enterococcus* sp. is of concern. While these bacteria are not human pathogens themselves, they are used as indicators of fecal contamination in environmental waters. Water quality criteria for ambient water, established by the US Environmental Protection Agency (US EPA) and adopted by North Carolina State water quality, use these bacterial species as indicators for the relative safety of water with regard to human pathogens and have set guidelines for the allowable concentration of bacteria in freshwater. North Carolina Department of the Environment

and Natural Resources Surface Water Standards state that the geometric mean of fecal coliform bacteria should not exceed 200cfu/100ml from five consecutive samples over the course of 30 days and no more than 20% of the samples should exceed 400 cfu/100ml (http://h2o.enr.state.nc.us/admin/rules/documents/Redbook2007_000.pdf). The US EPA further outline standards based upon *E. coli* and Enterococci (http://www.epa.gov/waterscience/beaches/local/statrept.pdf). These standards specify that the geometric mean (of five samples over 30 days) of *E. coli* should not exceed 126 cfu/100ml and for water used infrequently for recreation, no single sample concentration should exceed the upper 95% confidence limit (576 cfu/100ml). For Enterococci the geometric mean is not to exceed 31 cfu/100ml and no single sample concentration should exceed 151 cfu/100ml.

In the present study these water quality standards were exceeded in the majority of samples. The median concentrations of fecal coliforms (data not presented), *E. coli* and *Enterococcus* sp. in stream water (based on MPN estimation) were 1955 cfu/100ml 147cfu/100ml and 103cfu/100ml. Using North Carolina's fecal coliform standard as a guide, more than 75% of the samples exceed the maximum allowable fecal coliform standard (400cfu/100ml). Of the samples that did not exceed the ambient water standards, almost all of them were collected in the cool season, in which bacteria concentrations are expected to be lower. And even in this season there were some samples that approached or exceeded the allowable limits.

State and National water standards are based upon indicator bacteria rather than the pathogens themselves because of the expense and difficulties associated with analyzing pathogens in environmental waters. However, any detectable levels of human

pathogens in water accessed by people are of concern because of their known health effects. In the present study, concentrations of *Salmonella* were much lower in the surface water samples (pooled geometric mean =-1.5log$_{10}$cfu/100ml) than the concentrations of the indicator bacteria *E. coli* and *Enterococcus* sp. While there were some samples (10% of all stream water samples and 8 of 9 irrigation pond samples) in which no *Salmonella* were present, *Salmonella* were found in the majority of the surface water samples collected. Comparing the concentrations of *Salmonella* by sampling site, there were higher concentrations of *Salmonella* downstream of swine CAFOs compared to the upstream concentrations at these facilities ($p = 0.0390$). However, there was no statistically significant difference in *Salmonella* concentrations when comparing downstream samples of CAFOs and row crop farms.

The majority of ground water samples did not contain any *E. coli* or *Enterococcus* bacteria and none were positive for *Salmonella*.

Examining the samples with regard to antibiotic resistant bacteria, it is found that almost all of the of the bacteria isolated from swine waste were resistant to one or more antibiotics including 99% of *E. coli* and Enterococci isolates and 83% of *Salmonella* isolates. In all genera and species, the frequency of resistant bacteria is much lower in the water samples compared with that in the animal waste ($p < 0.0001$ in all species). In water samples 88% of *Salmonella* sp. isolates, 61% of *E. coli* isolates and 0.5% of Enterococcus sp. isolates were not resistant to any antibiotics; 13% of the Enterococcus isolates were not resistant to any clinically significant drugs.

Because many species of *Enterococci* have intrinsic resistance to one or more antibiotics, a more reliable comparison of *Enterococci* isolates to Gram-negative bacteria

isolates was attempted by consideration of resistance to two or more antibiotics. On this basis, *Enterococcus* isolated from water had a much lower incidence of multi-drug resistant isolates than those from animal waste, with 39% of Enterococci from water having resistance to two or more human clinically significant drugs compared with 91% of water isolates from swine waste samples.

When comparing frequency of resistance in bacteria isolates from water up and downstream of the farms within farm type, no statistically significant differences were seen for those from row crop farms or swine agriculture facilities. Furthermore, when comparing frequency of single or multiple resistance in *E. coli* and *Enterococcus* sp. between farms types (comparing downstream samples), there was no difference in the proportions of antibiotic resistant bacteria of either species.

The proportion of antibiotic resistant *Salmonella* was found to be significantly higher downstream of animal agriculture facilities than downstream of row crop farm ($p=0.0144$). But, since there was no difference in the upstream and downstream concentrations or the incidence of resistant *Salmonella*, the higher incidence of resistant *Salmonella* cannot be conclusively attributed to an impact from the animal agriculture facility.

One potential reason that no differences were seen in bacteria concentrations or their resistance frequencies between the up and downstream water samples of the study farms could be very high animal agriculture density in the region (figure 7.2 and 7.3 (zoom image)). This high animal agriculture farm density may have resulted in high background levels of the bacteria in ambient waters. The high background of bacteria may have masked the detection of any potential individual inputs by the study farms.

There are thousands of animal operation permits in North Carolina and many of those are swine facilities in the eastern region of the State (NConeMAP). While many of the study sites were not in the counties with the highest densities of farms, there are several study sites in areas having many other CAFOs including the study farms in Greene county. This county, while not having the highest density of swine farms in the state, does have a large number of CAFO facilities.

Efforts were made to spatially isolate farms (animal agriculture and row crops) in order to prevent environmental bacterial cross contamination from one farm to the other. However, given the high farm densities in the study geographical areas and the need to limit site selection to those farms with non-ephemeral water bodies flowing through or adjacent to a study farm, this was not always possible.

Figure 7.2: Sites of Animal operation permits (green squares) and Swine Lagoons (red triangles) in North Carolina (NConeMAP data)

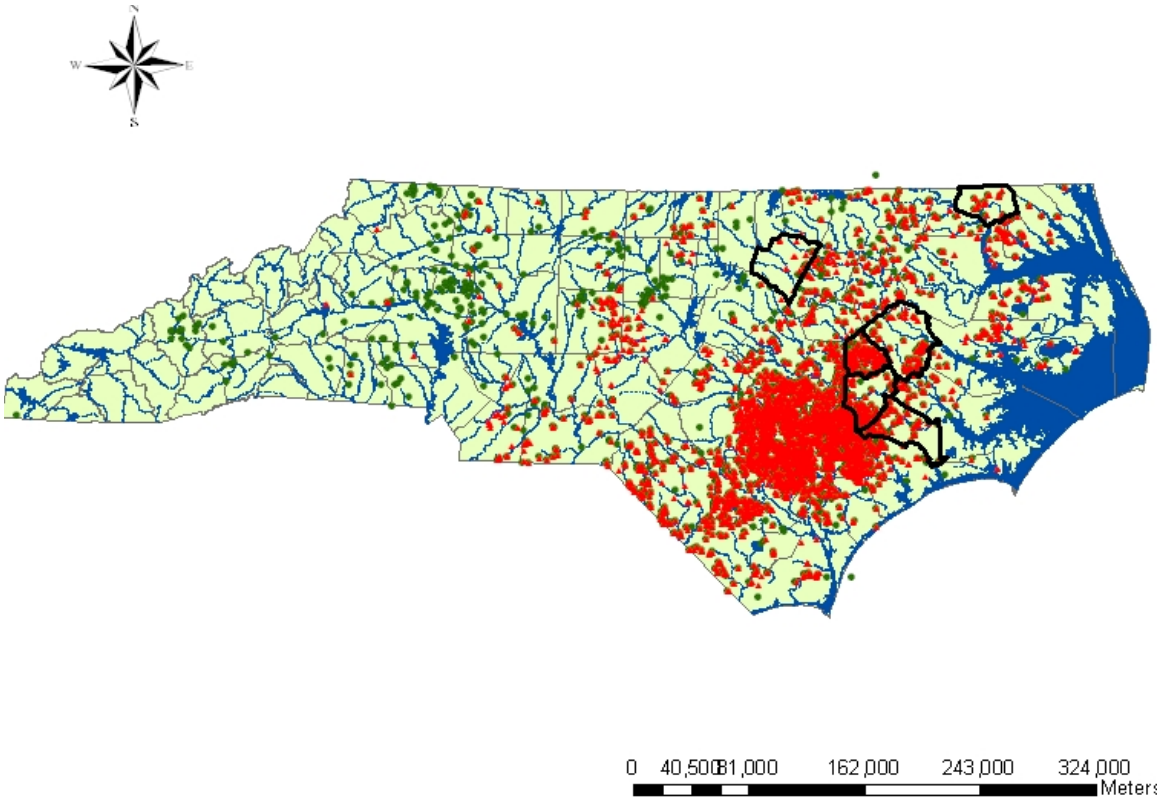
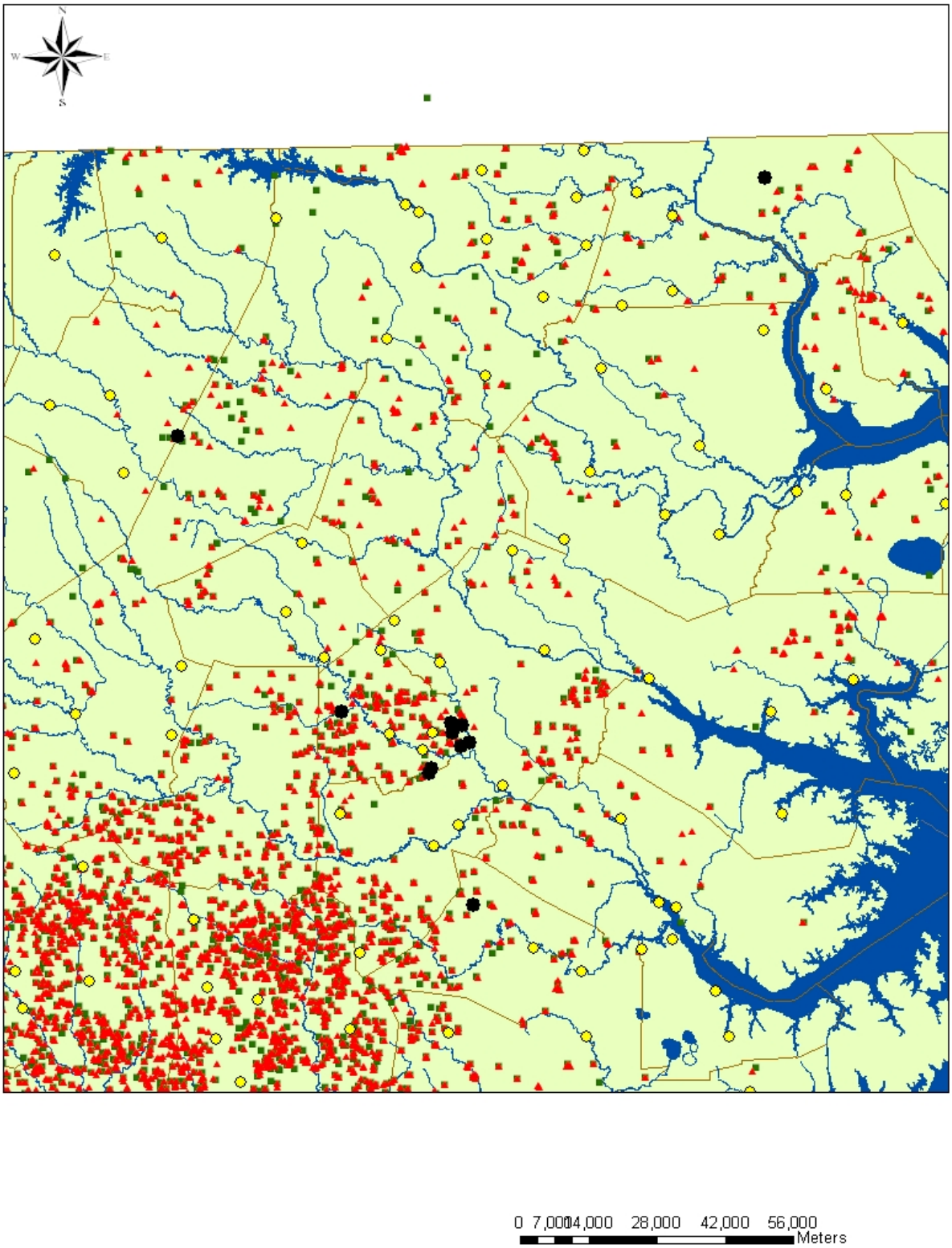


Figure 7.3: Animal Agriculture Study Sites (large black circles) in Relation to Other Animal Agriculture facilities (all permits green squares, swine lagoons red triangles) and Human Wastewater Treatment Facilities (yellow circles)



Non-animal agriculture (row crop farm) sites (not on maps) were generally more remote from animal agriculture sites than the animal agriculture (swine farm) sites of the study. In order to be able to sample in the same general geographical area as the animal agriculture facilities in the study, it was logistically impossible to choose non-animal agriculture sites having no impact from non-study animal agriculture facilities. As a result, it is possible that background levels of enteric bacteria in the vicinity of the study row crop farms were also elevated and therefore, had an uncontrollable confounding effect on the overall comparison between study farm types.

Environmental Bacteria Antibiotic Resistance Profiles

Many of the bacteria isolated from the environment of study farms were resistant to one or more antibiotics. The antibiotic for which there was the most resistance in either Gram positive or Gram negative bacteria was tetracycline. Of the bacterial isolates resistant to one or more antibiotics, 284 *E. coli* (84%), 108 *Salmonella* (87%), and Enterococci sp. 362 (76%) were resistant to tetracycline.

In addition to tetracycline, resistance to several other drugs was also common. For the Gram negative bacteria, bacterial resistance to the antibiotics ampicillin, sulfafisoxazole, trimethoprim/sulfamethoxazole, kanamycin and streptomycin were the most prevalent. Of the resistant *E. coli*, resistance percentages were 37% to ampicillin, 30% to sulfisoxazole, 18% to kanamycin, 18% to trimethoprim /sulfamethoxazole and 17% to streptomycin. Of the resistant *Salmonella*, resistance percentages were 69% to ampicillin, 73% to sulfisoxazole, 25% to streptomycin and 8% to kanamycin.

Resistance of Gram-negative bacteria, such as *E. coli* and *Salmonella,* to the suite of drugs consisting of tetracycline, ampicillin, sulfa drugs, streptomycin, and kanamycin is common. Several studies in the United States and worldwide have documented high frequency of tetracycline resistance among bacteria isolated from various settings including human waste treatment facilities, clinical specimens, environmental media such as water, produce and animal wastes (Esiobu, N. et a., l 2002, Schroeder, C.M. et al., 2002, Johnston L.M. and Jaykus, L., 2004, Sayah, R.S., et al 2005, Gebreyes, W.A. 2006, Stine, O.C. 2007). These documented frequencies of tetracycline resistant isolates ranging from about 20% to 85%. In many of these studies tetracycline-resistant bacteria were also resistant to ampicillin, sulfa drugs, streptomycin, and kanamycin.

The occurrence and frequency of resistance to combinations of drugs is of special interest because it suggests that these resistance genes are genetically co-located and remain associated as a group when transferred from one bacterium to another. In this study the Gram-negative bacteria that were resistant to more than one drug were most commonly resistant to tetracycline, ampicillin and/or sulfafisoxazole. Many multi-drug resistant bacteria were also resistant to kanamycin, streptomycin and/or gentamicin. As with the high frequencies of resistance to these drugs individually, resistance to this combination of drugs in Gram negative bacteria was not rare. Of the *E. coli* isolates resistant to 3 or more antibiotics, 44% had resistance to tetracycline, sulfisoxazole and ampicillin. Of those resistant to 5 or more drugs 82% were resistant to all three of these drugs. Almost all of the multi-drug resistant *E. coli* were resistant to at least two of these three drugs.

This finding is consistent with previous studies that have identified resistance to these combinations of drugs and have identified plasmids that contain genes promoting resistance to these drugs on a single cassette or chimeric genetic element (Leverstein- van Hall, M.A. et al 2003, Gebreyes, W.A. et al., 2004, Gebreyes, W.A. et al., 2006).

As in Gram-negative bacteria, there was a high occurrence of mono- and multi-drug resistance in the *Enterococcus* isolated from various sources. Resistance profiles and frequencies of *Enterococcus* sp. were complicated by the fact that many species have intrinsic resistance to one or more antibiotics. The break points for aminoglycoside (including streptomycin and gentamicin) resistance are different in Enterococci than the Gram-negative bacteria, due to intrinsic Enterococcus resistance to low levels of these drugs. Other drugs to which certain Enterococcus species have intrinsic resistance at low levels include β-lactam antibiotics such as penicillins and ampicillin, macrolides, lincosamides and streptogramin B (Facklam, R.R., et al., "the Enterococci", 2002). Almost all *E. faecalis* have intrinsic resistance to streptogramin A compounds which enables resistance to quinupristin/dalfopristin (a streptogramin A and streptogramin B combination) (Kak, V. and Chow, J.W., "the Enterococci" 2003). Therefore, when "resistance" to these drugs is presented in this study is implied to be high level resistance, beyond that attributable to intrinsic resistance.

Different *Enterococcus* species have the tendency to acquire and maintain resistance to specific antibiotics. For example, the majority of *E. faecium* isolates are ampicillin resistant while less than 2% of *E. faecalis* demonstrate such resistance (Klare, I. et al., 2003). Likewise, lincomycin resistance occurs in *E. faecium* but rarely in *E. faecalis* (Kak, V. and Chow, J.W. 2003).

There are some antibiotics for which most Enterococcus, regardless of species, have acquired and maintained resistance. For example, in clinical settings more than 60% of isolates have been found to have tetracycline resistance (Kak, V. and Chow, J.W. 2003).

In this study, Enterococci environmental isolates exhibited a high frequency of resistance to several different antibiotics, including tetracycline (as mentioned earlier) at 76% (362 isolates). Of these tetracycline-resistant isolates, 79% were resistant to quinupristin/dalfopristin, 55% were resistant to erythromycin, and 33% were resistant to high levels of streptomycin. For the veterinary drugs, 94% of those isolates resistant to at least one clinically significant drug were resistant to lincomycin at the MIC_{90} breakpoint (>32µg/ml), 56% were at or above the MIC_{90} for tylosin (>32µg/ml), and 41% were at or above the MIC_{90} for flavomycin (>16µg/ml),

There were species differences in Enterococcus resistance to the different drugs. For quinupristin/dalfopristin resistance, almost all *E. faecalis* were resistant while less than 40% of the *E. faecium* were resistant. For erythromycin, almost 70% *E. faecium* were resistant while about 50% *E. faecalis* were resistant. Flavomycin resistance was 90% in *E. faecium* and less than 10% in *E. faecalis*.

The high frequencies of antibiotic resistance of *Enterococcus* sp. found in this study are similar to those found in previous research. Buscani, L., et al. (2004) found high levels of tetracycline resistance in Enterococcus isolates collected from raw meat products, farm animals and human samples in Italy. Butaye, P. et al. (2001) reported high frequencies of tylosin resistance among Enterococcus isolates from a variety of farm animals and pets, with tetracycline resistance in all the isolates tested. A notable

difference in this present study compared with many others is the lack of vancomycin resistance among the environmental and human Enterococci isolates. None of the environmental isolates tested were resistant to vancomycin at the breakpoint of ≥32μg/ml. One isolate had intermediate resistance that can be attributed to its species, *E. casseliflavus*, which is known to have intrinsic low level resistance to vancomycin mediated by genes within the chromosome.

Human Isolates Antibiotic Resistance Profiles

During this study a total of 87 people submitted 578 human stool specimens, with submission of one to twelve specimens per person over a twelve month period. The year of stool sample submission was concurrent with the environmental sampling on and around the farms in their neighborhood. Each specimen was prescreened for at least minimal resistance to one of five clinically significant antibiotics. Isolates that grew on plates with low levels of antibiotics were then analyzed for their resistance to a suite of antibiotics, as were the environmental isolates.

Of the 578 specimens submitted, 285 (49%) did not yield any bacteria resistant to low levels of screening antibiotics. The other 293 samples that yielded bacteria resistant to low levels of screening antibiotics provided 148 *E. coli* isolates and 265 Enterococcus isolates. There were 106 specimens that yielded at least one *E. coli* isolate and 200 specimens that yielded at least one *Enterococcus* isolate. Some specimens yielded both of these bacterial and/or multiple isolates of one species. Upon further analysis of initial isolates, three *E. coli* that grew at the prescreening antibiotic concentrations did not have resistance to any of the 15 antibiotics at the NCCLS breakpoint concentrations. All other

E. coli and *Enterococcus* isolates obtained were resistant at the NCCLS breakpoint concentrations to at least one of the antibiotics analyzed.

As with the environmental samples, tetracycline resistance was the most frequent, occurring in 70% of the isolates. Ampicillin and sulfisoxazole resistance was common with 66% and 55% of the isolates resistant, respectively. Resistance to the streptogramins, (gentamicin and streptomycin), Naladixic Acid and Ciprofloxacin was also frequent with at least 30% of the isolates resistant to these drugs.

The high rate of resistance to ciprofloxacin observed in bacteria of this study is of particular concern because this drug is used to combat infections in humans such as Salmonellosis (Molbak, K., et al., 2002). Ciprofloxacin resistance of bacteria isolates was seen in 10 people, which is more than 11% of the study population. Such a high rate of ciprofloxacin resistant bacteria in healthy participants may be evidence rapid emergence of resistance to this drug. In 1992 and 1994 greater than 99% of all clinical isolates tested in the United States, Canada and the United Kingdom were susceptible to ciprofloxacin (Thomson, C.J. 1999). In Denmark, less than 1% of isolates from healthy human volunteers were found resistant to ciprofloxacin in 2003 (DANMAP. 2003). These low frequencies of ciprofloxacin resistance in clinical isolates from people of previous studies are different from the 30% of isolates (11.4% of people) resistant to ciprofloxacin seen in this present study.

In the last decade, studies have provided evidence of increased ciprofloxacin resistance in *Salmonella* species. This resistance has been attributed to the emergence of the DT104 serotype (Threlfall, E.J. et al 2000). In our study however, *Salmonella* were not isolated from any of the human specimens. The extent to which resistance to

ciprofloxacin seen in the present study was related to the emergence of this strain of ciprofloxacin-resistant S*almonella* documented in previous and the transfer of the plasmid to the *E. coli* isolates is unknown.

It has also been suggested that an increase in bacteria resistance to ciprofloxacin may be attributable to the use of fluoroquinolones in animal agriculture (Molbak, K. et al., 2002). However, in the present study, none of the environmental isolates including those from swine and cattle waste were found to be resistant to ciprofloxacin. Of the 10 people that had ciprofloxacin resistant bacteria, 6 of them (accounting for 37 of the 47 ciprofloxacin positive isolates) were not associated with animal agriculture. Furthermore, none of the individuals with ciprofloxacin resistant isolates were animal agriculture growers, who are the people one would expect to be at higher risk of acquiring resistant bacteria from the animals or animal wastes than those with no such contact, if animals or animal wastes were indeed the source of ciprofloxacin-resistant bacteria.

In addition to antibiotic resistant *E. coli* isolates, there were many mono- and multi-drug resistant *Enterococci* isolated from human stool samples As with *E. coli*, tetracycline resistance was very common among the human *Enterococcus* isolates, 87% of which were resistant to tetracycline. Accounting for the number of specimens that did not have any resistant isolates, approximately 30% of specimens submitted in this study had tetracycline resistant *Enterococci*. Because Kak, V. and Chow, J.W. (2003) report that at least 60-65% of clinical isolates are resistant to tetracycline, the 30% of specimens with *Enterococci* isolates resistant to this drug in this present study is not unusually high.

Resistance to quinupristin/dalfopristin and erythromycin is also prevalent among the human isolates at 63% and 29%, respectively. As mentioned previously,

quinupristin/dalfopristin resistance is often species-associated and this pattern was seen in the Enterococci isolated from human specimens in this study. Almost 100% of the *E. faecalis* isolates are resistant to this drug while only about 15% of the *E. faecium* isolates were resistant.

Human *Enterococcus* isolates also had resistance to drugs used in veterinary medicine, with 70% to lincomycin, more than 40% to flavomycin and more than 25% to tylosin tartrate. Resistance to these three drugs is also species-dependent. Therefore, some of the observed resistance to specific drugs could be intrinsic in one species or another. For Example, almost 100% of the *E. faecium* isolates collected from human stool samples were resistant to flavomycin. In contrast, less than 10% of the *E. faecalis* isolates had resistance to this drug. This discrepancy by species could indicate that resistance to flavomycin in *E. faecium* is intrinsic but that resistance to this drug in *E. faecalis* is an acquired trait.

Resistance to tylosin in human isolates is of concern as this drug is exclusively used in veterinary medicine. One or more tylosin resistant isolates were found in 24 people. Of these, 15 were associated with animal (swine) agriculture and 9 were associated with row crop farms. However, while it is possible that people are acquiring bacteria resistant to this drug from the animal agriculture facilities, there are other potential exposures and/or reason for this resistance.

First, as tylosin is a macrolide antibiotic, it is possible that resistance in these bacterial isolates from people is due to cross resistance generated by selective pressure of other macrolides such as erthyromycin, which is commonly used in human medicine. Furthermore, there are some mechanisms of resistance to macrolide antibiotics that are

linked to resistance to lincosamides and streptogamin B. There are many antibiotics from these classes used in veterinary and human medicine, and such use may have promoted resistance mechanisms that would be effective against tylosin as well.

A second possible explanation for the relatively high incidence of tylosin resistance could be a consequence of exposure via pets. Butaye, P., et al. (2001) found that *E. faecalis* and to a lesser extent *E. faecium* isolates from various pets had a high incidence of tylosin resistance. They found that while tylosin resistant *E. faecium* was more common among farm animals than pets, tylosin resistant *E. faecium* was isolated from feces in all of the pets varieties sampled. Of *E. faecalis* isolates collected in their study, Butaye et al. found all animals, farm animals and pets, had high prevalence of tylosin resistance and there was no significant difference in the frequency of tylosin resistant *E. faecalis* isolated from pets as compared with those isolated from farm animals.

In our study, fecal matter was not collected from pets of the participants. However, comparisons can be made regarding the people who harbored tylosin resistant bacteria and having pets. 80% of the participants have pets; 67% report having dogs, 37% have cats and 6% have birds. Of those 24 individuals that harbored one or more Enterococci resistant to tylosin, 20 of them report owning pets; 10 of these have dogs and 15 have cats and 23 have birds (some of the birds include a rooster, or peacocks not considered pets). While these data do not conclusively establish pets as a source of the tylosin resistant bacteria in humans, it does allow for another possibility of exposure.

Comparative Analyses of Antibiotic Resistance in Bacteria of Different Sources

Prevalence of *E. coli* resistant to one or more antibiotics was higher in animal waste samples than in water samples or in human stool specimens. Drug resistant *E. coli* was found in 86.5% of animal waste samples (with resistance frequencies of 99% in isolates from swine waste and 43% in isolates from cattle manure), 36 % of ground and surface water samples and 18% of human fecal samples. The prevalence of resistance among the swine waste samples was higher than in the other samples, the magnitude of resistance (i.e. the number of antibiotic to which an isolate is resistant) was not higher in the swine waste samples compared to the other samples.

Overall, the resistant *E. coli* isolates collected from people in this study had resistance to more antibiotics than those isolated from environmental samples. The most antibiotics to which any of the animal waste isolates were resistant was 9 (2 isolates), in stream water there was one isolate resistant to eight antibiotics and in human isolates there were 7 isolates (nearly 5%) that were resistant to 10 different antibiotics. Comparing median values (based on the *E. coli* isolates from each sample type resistant to one or more antibiotics), 50% of the isolates collected from animal wastes were resistant to 2 or more drugs, in water the median value was 1 drug and in people the50% of the isolates were resistant to 4 or more drugs Furthermore, when comparing proportions of multi-drug resistance in human isolates to those collected from swine waste, it was seen that more human isolates are resistant to multiple antibiotics ($p = 0.0015$).

As with the *E. coli* isolates, multi-drug resistance in Enterococcus was more frequent in environmental samples than in human samples. However, unlike *E .coli*,

there was no difference in the proportions of isolates resistant to multiple antibiotics based on the source of the sample.

Comparing the frequency and magnitude of drug resistance in people according to the farms with which they were associated, there was no difference in the frequency distributions of the multi-drug resistant Enterococcus ($p = 0.650$) nor the magnitude of resistance in the isolates ($p = 0.8897$). Additionally, there was no difference in the occurrence or frequency of *Enterococcus* resistance to veterinary drugs in these populations ($p=1.000$).

A statistically significant difference was found among the two farm type exposure groups for resistance of *E. coli* isolates. Overall, there was a higher proportion of resistant *E. coli* isolates collected from specimens submitted by people associated with CAFOs than with row crop farms. However, there was a significantly higher frequency of multi-drug resistant E. coli isolates in specimens from people associated with row crop farms than with swine farms. Furthermore, the proportion of *E .coli* isolates resistant to 4 or more drugs was significantly higher among people associated with row crop farms ($p=0.0007$). Thus, people associated with swine farms had a higher risk of having bacteria resistant to at least one antibiotic, but people associated with row crop farms harbored bacteria with resistance to more antibiotics.

Phenotypic Links between Environmental and Human Bacterial Isolates

Overall, conclusive links between the bacteria isolated from the environment and those from people living near or working on farms could not be established. Many of the bacteria had similar antibiotic resistance patterns, such as isolates with mono-

resistance solely to tetracycline, or isolates with multi-drug resistance to ampicillin, tetracycline and sulfisoxazole (Gram-negatives) or macrolide-streptogramin –lincosamide combinations (Enterococci). These multi-drug resistance patterns are commonly seen in resistant bacteria and all of them have been associated with animal agriculture as well as non-animal agriculture exposures. Furthermore, in the environmental analyses of this study, the bacterial concentrations as well as the prevalence of antibiotic resistant bacteria were not found to be different upstream or downstream of the study farms nor by study farm type. As a result, the specific sources contributing to antibiotic resistant bacteria in the stream water were not be elucidated in this study. As previously mentioned, these farms may have contributed to the total and antibiotic-resistant bacterial load of farm waters, but that contribution was masked by the relatively high background concentrations of such bacteria, as documented by total and antibiotic-resistance bacteria concentrations in upstream water samples.

There was a statistically significant difference between the prevalence of antimicrobial resistant *Salmonella* downstream of animal agriculture compared to those downstream of row crop farms. However, there were no cases in which people in the communities were found to harbor *Salmonella*. Therefore, at the time of the study, potential exposures to Salmonella in water did not appear to constitute a risk to people in the community, based on the limited human Salmonella surveillance data collected as voluntarily submitted monthly stool samples during a 1-year study period.

One of the goals of this project was to ascertain the extent to which people's exposure to water that could be impacted by animal agriculture was associated with acquisition of antimicrobial resistant bacteria originating from the two different farm

types studies, swine and row crop. However, an impact by the farms on the bacterial concentrations in farm ambient waters could not be established. Additionally, bacterial isolates from human stools could not be conclusively linked to exposures from the farms with which the individuals were associated. Hence, it can not be concluded that farm exposure to water (or to swine waste on swine farms) was a significant route of exposure to and resulting transmission of resistant bacteria originating on farms. It must be noted however, that the small scale of this project may have not allowed for detection of some of these potential impacts. Furthermore, it is possible, that molecular analyses of these bacteria and their resistance traits may provide more conclusive identifications and insights into the sources of patterns of resistance and thereby, provide a better linking or tracking of the source of the bacteria in humans where phenotypic analyses could not provide this.

Risk of Carriage of Antibiotic Resistant Bacteria

A link of the antibiotic resistant bacteria found in humans to the farms with which they were geographically associated was not established on a microbial source tracking or molecular epidemiological basis in this study. Nevertheless, it is important to understand the extent to which individuals harbor resistant bacteria and establish if there is any relationship of their antibiotic resistant bacteria status to their environment, including association with animal agriculture or row crop farms. To this end, this study examined the risk of harboring antibiotic resistance bacteria if a person lived near or worked on an animal (swine) agriculture facility compared with those who were associated with row crop farms.

A total of 87 people submitted at least one fecal specimen, of whom 47 (54%) were associated with animal agriculture and 40 (46%) were associated with row crop farms (40 people). A person was considered positive for antibiotic resistance carriage if one or more of the specimens submitted contained at least one antibiotic resistant-bacterium. Of the 87 people, only 16 (18%) submitted specimens that did not yield resistant bacteria. Of these 16 people from which no antibiotic-resistant bacteria were isolated from submitted stools, 12 (75%) were associated with row crop farms and 4 (25%) were associated with animal agriculture facilities.

Log-linear regression was used to estimate the risk of carriage of antibiotic resistance when living near or working on animal (swine) agriculture facilities compared with those who live near or work on row crop farms. This model estimates a Risk Ratio (RR). There are some limitations to using this model because the outcome in this study was not rare. In such a situation it is more appropriate to use the log-linear model to estimate an odds ratio rather than the logistic model. In situations for which the outcome is rare, the odds ratio approximates the risk ratio. However in situations where the outcome is not rare, such as in this study, a logistic model trends to over estimate the effect (Rothman and Greenland, "Modern Epidemiology", 1998).

Using a crude model for only exposure (farm association) and outcome (positive for carriage), the RR is 1.31 (1.05-1.63). As the 95% confidence interval does not include the null value of 1, this effect is considered to be significant. The RR of 1.31 indicates that those who are associated with animal (swine) agriculture facilities are 0.31 times more likely to harbor antibiotic resistant bacteria than those associated with non-animal agriculture (row crop) farms.

There are other factors that may have an impact on this estimate and therefore need to be considered for their possible effect on the estimation. These factors include demographic variables such as age, gender and income, as well as other potential exposures, such as taking various medications, foreign travel, chronic disease, hospital/doctors visits or having pets. A total of 17 different variables were considered and analyzed for their overall impact on the model. Five were found to have an impact on the overall model based on a 10% change in estimate. These included age, taking antibiotics, chronic illness, having pets and using a well as a drinking water source. Using a backward elimination approach and the 10% change in estimate criterion, it was determined that only three of these variables were, in fact, required in the final model: drinking water source, antibiotic usage and having pets. Therefore the final model was:

$$\textbf{Prob(outcome)} = \alpha + \beta_1\textbf{(exposure)} + \beta_2\textbf{(drinking water source)} + \beta_3\textbf{(antibiotic use)} + \beta_4\textbf{(pets)} \qquad \textbf{eqn 7-1}$$

Using this model, the final risk ratio is estimated to be 1.42 (1.17-1.72). Again as the confidence interval does not cross the null value of 1.0, it is considered statistically significant. This risk estimate suggests that even when considering potential confounding on exposure, there is still a higher risk of carrying antibiotic resistant bacteria if someone lives near or works on animal (swine) agriculture facilities compared with those associated with row crop farms. In this study people associated with animal (swine) agriculture are 1.42 times more likely to harbor antibiotic resistant bacteria than people associated with row crop farms.

It is noteworthy that being a farmer (or not) had no an effect on the regression model, i.e. including "farmer" as a binomial covariate in the regression model did not result in a 10% change in the regression coefficient. This suggests that risk carriage of resistant bacteria was not increased for those who are farmers as compared with neighbors. Previous studies, however, have indicated that those who work with animals tend to have a higher incidence of carriage of antibiotic resistant bacteria compared with non-farmers living in the same areas (Levy, S.,1978, Aubry-Damon, H., et al. 2004). In this present study when examining swine farmers as the "exposure" compared with row crop farmers, the animal agriculture growers did have a higher risk of carriage of antibiotic resistance in stool bacteria than row crop farmers, however this effect was not considered significant 1.93 (0.90 – 4.13) as the 95% confidence interval included the null value. In this estimate as well as in the overall model it is likely that the effect of being an animal agriculture grower is not significant due to the very low sample size. The estimated risk ratio of 1.93 being greater 1.0 suggests a possible effect in the direction of greater rather than lower risk of swine farmers for antibiotic resistant bacteria presence in stool samples. Only 23 of the 87 people who submitted stool specimens were farmers (14 animal agriculture farmers and 9 row crop farmers). Given these low numbers, there is a lack of precision in the estimate and therefore making it difficult to detect a statistically significant effect.

While there appears to be a higher risk of antibiotic resistant bacteria carriage among those associated with animal agriculture than those associated with row crop agriculture, the source of these antibiotic resistant bacteria is uncertain. As mentioned earlier, there was no molecular epidemiological or conclusive microbial source-tracking

evidence to link the antibiotic resistant bacteria found in people to their exposure to contaminated ground or surface water in their environment. Other potential sources of or exposure routes for antibiotic resistant bacteria that could contribute to increase in risk of presence in exposed people include soil, produce and both indoor and outdoor air (Nwosu, V.C., 2001, Esiobu, N. et al 2002, Senegelov, G. et al, 2003, Johnston L.M. and Jaykus, L. 2004, Gibbs S.G. et al 2006) . It is possible that those who live near the animal agriculture facilities are exposed to bacteria in the environment by these other environmental routes of exposure besides the water route analyzed in this study. It is also possible that people are exposed to and can acquire resistant bacteria from the swine CAFO environment, but they are bacterial species not studied in this research. Because bacteria can exchange resistance trait among and between different bacterial species, the trait(s) may have been exchanged between the *E. coli and Enterococcus* analyzed in this study and other bacteria associated with farm environments and with the people of those environments.

Gibbs, S.G. et al. (2006) analyzed airborne bacteria in the vicinity of swine farms and found antibiotic resistance in them as far as 150 meters away (the farthest distance studies). The most prevalent species in these air samples was *Staphlococcus aureus*. Several bacterial species in soil have also been found to have antibiotic resistance. In a review article, Nwosu, V.C. (2001) cites several studies in which different bacterial species including *Streptomyces, Bacillus, Aeromonas* and *Enterobacter* found in soils were resistant to a variety of antibiotics including erythromycin and other macrolides. Nwosu, V.C. also discussed the rapid degradation of antibiotics in soils and suggests that the high prevalence of resistant bacteria in soil is likely do to selective pressure from

heavy metal resistance as oppose to residual antibiotics or residues in the soil. While these studies did not examine soils surrounding animal agriculture facilities they did provide evidence that soil can contain resistant bacteria and that these bacteria can be transferred to people.

Senegelov, G., et al. (2003) examined the impact of the spread of swine manure slurry on Danish farmland. High levels of resistance genes to tetracycline, streptograms and aminoglycosides in the were found in Gram-negative bacterial isolates collected from soils amended with this slurry, which may provide a reservoir of resistance genes that could then create increased risks of exposure to resistant bacteria to people in the area.

The higher risk of antibiotic resistant bacteria carriage in people associated with swine farms is the possible presence of residual antibiotics in their environment, either from the animal agriculture facilities (e.g., swine waste) or from other nearby sources such as swine feed, swine drinking water and human waste water treatment facilities or septic systems. Chee-Sanford, J.C. et al. (2001) reported residual tetracycline resistance genes in swine lagoons and in groundwater underlying these lagoons. Hirsch, R., et al. (1999) found residuals to several antibiotics in sewage treatment plant effluents and in stream waters. Furthermore, it may be possible that environmental bacteria are being exposed to these residuals and acquiring resistance genes. People could then be exposed to these resistant bacteria and acquire them in their gut flora. Further research into antibiotic residuals in water and other environmental media and their impact on the presence and persistence of antibiotic resistance genes in environmental bacteria should be conducted,

Conclusions

This was a pilot study and the overall sample sizes were relatively low; concentrations of enteric bacteria and the frequencies of antibiotic resistance were assessed in only 11 swine farms and 6 row crop farms of the thousands of farms that are present in Eastern North Carolina. Only 87 people participated in the human component of this study. While efforts were made to ensure similarities between the study population and the general population in Eastern North Carolina, it cannot be concluded that the results of this study can be generalized to the entire region nor do the bacterial concentrations found in these farms indicate what may be found in or around all farms in the region.

In order to assess the potential impacts of CAFOs on environmental water and human health effects for those who live near or worked on the farms, this study addressed four different components: 1) Are there enteric bacteria present in CAFOs (specifically in animal waste) and at what concentration? 2) Are antibiotic resistant enteric bacteria present in CAFOs and at what frequency? 3) Are enteric bacteria from the farms affecting environment, specifically surrounding environmental waters, and if so at what are the bacterial concentrations and frequencies of antibiotic resistance in the bacteria found in the environmental water? And finally, 4) What is the frequency of antibiotic resistance, as well as the proportion of multiple antibiotic resistance, in people who live near or work on CAFOs compared with those who are associated with row crop farms?

High concentrations of single- and multi-drug resistant enteric bacteria, specifically *E. coli*, *Salmonella* sp. and *Enterococcus* sp., were present in animal waste on eleven swine farms studied in eastern North Carolina. Almost all of the bacteria isolates

collected from swine waste samples were resistant to at least one drug, including 83% of *Salmonella* sp., which are frank pathogens. Cattle manure on swine farms also contained antibiotic resistant bacteria and *Salmonella*. The high concentrations of antibiotic resistant bacteria found in wastes on these farms suggest that these swine CAFOs are a potential source of exposure to antibiotic resistant bacteria.

Examining ground and surface waters surrounding the CAFOs and surface water surrounding row crop farms revealed that enteric bacteria were often present (in much lower concentrations than those found in animal waste) in surface waters but rarely or not at all in ground water samples. Furthermore, the frequency of antibiotic resistant enteric bacteria in the water samples was much lower than that of the bacteria isolated from animal waste.

Of the bacteria isolated from water, 61% of *E. coli* and more than 88% of *Salmonella* had no antibiotic resistance. About 60% of the *Enterococcus* sp. isolates found in water were not resistant to any antibiotics or resistant to only one drug of human clinical significance. Due to intrinsic resistance, Enterococci isolates from water were more likely to have resistance to at least one antibiotic. However, multi-drug resistance (specifically resistance to two or more clinically significant drugs) is more likely to result from acquired resistance traits rather than intrinsic resistance.

Of those isolates that did have antibiotic resistance traits, phenotypic links between the bacteria found in the environmental water and the farms were not established. Therefore, the source of the resistant bacteria in the environmental waters was not identified and could not be attributed to the farms.

Comparing concentrations and antibiotic resistance frequencies by site (up and downstream of CAFOs and row crop farms) revealed that enteric bacteria concentrations in stream water samples were not statistically significantly different from one another, and therefore, it is concluded that a detectable impact on environmental waters by the CAFOs is minimal if at all. Two factors could contribute to the lack of ability to detect an impact of individual swine farms on concentrations and antibiotic resistance properties of enteric bacteria on water: (1) overall high background levels of bacteria with antibiotic resistance possibly emanating from the high numbers and densities of animal agricultural operations in the study areas, and (2) a lack of consideration of other environmental sources of antibiotic resistant enteric bacteria on farm environments, such as soil, vegetation and air.

It must be noted that samples were not taken during periods of land application of swine waste lagoon liquid or after extreme weather events such as floods or hurricanes. Although these events could result in greater presence of enteric bacteria in ambient waters, such potential impact of these farms on the presence and levels of enteric bacteria, including pathogens and those with antibiotic resistance, were not considered.

Examining the potential human health effects of resulting from living near or working on these swine CAFOs, it was found that in the study population those associated with the swine CAFOs were more likely to harbor antibiotic resistant enteric bacteria than those living near or working on row crop farms. However, it must be noted that the bacteria found in the people could not be linked to the bacteria found in the environmental waters, nor the animal wastes, therefore the source of the resistant bacteria in people is uncertain. Accounting for potential confounders, a risk ratio (RR) of 1.42

(95% CI = 1.17-1.72) was estimated for people associated with swine farms compared to people associated with row crop farms. This estimate is statistically significant with relatively good precision.

While this result reveals that people associated with CAFOs are more likely to carry antibiotic resistant bacteria than those associated with row crop farms, it does not address the magnitude (i.e. the number of antibiotic to which the isolates are resistant) of the resistant isolates found in the two study populations. When comparing proportions of isolates with multiple drug resistance in the two exposure groups, it was found that those people associated with row crop farms harbored isolates with more resistance traits than those isolates from people associated with CAFOs. Therefore, while people associated with CAFOs are more likely to harbor at least isolates with resistance to at least one drug, the people associated with row crop farms harbor bacteria that are potential more dangerous.

Given these conflicting results, and the fact that bacteria found in the environmental water and humans could not be conclusively linked to the farms, it cannot be concluded from this study that association with swine CAFOs results in higher overall risk of antibiotic resistance.

This study was a small scale pilot study and lacks the statistical power and representativeness to detect impacts of swine farms and enteric, antibiotic-resistant bacteria, possibly from these farms, on people associated with these farms and on the nearby aquatic environment. However, the study results provide some new insights into the possible role of animal agriculture on the occurrence and environmental dissemination of antibiotic resistant bacteria. This study also provides new information

regarding overall carriage burden of antibiotic resistant bacteria in people living in this rural region of eastern North Carolina and working in CAFO environments.

Further Research

As this was a pilot study, a larger scale project that examines not only the possible role of water but other potential routes of exposure to antibiotic-resistant and pathogenic enteric bacteria, including environmental, person-to-person and animal-to-person routes, should be conducted. With a larger number of farms and human participants, much more rigorous and representative analyses can be conducted. Expanded and improved analyses would include more robust risk analysis including examining the effects of household and neighborhood clustering on the overall outcome. Furthermore, an increase in the number of farms and study participants would achieve greater statistical power.

In addition to larger scale studies, additional and more informative data on the properties of the bacteria isolates that have already been collected and those that could be collected in future studies should be obtained. Robust molecular analyses such as multi-locus sequencing typing of the bacterial genomes for speciation and genetic characteristics of antibiotic resistance traits may provide greater insight with regard to the sources of the bacteria found in the environmental waters and in the people. These analyses would also yield information regarding the specific genes that are enabling resistance within the bacteria. There are several different mechanisms by which antibiotic resistance is achieved. Analysis of the resistance traits often can provide insights into how resistance to different antibiotics was acquired and how it may be transferred. Different microorganisms may utilize different mechanisms for resistance to

the same drug or to multiple drugs that constitute a set of resistance properties. By genetic sequencing, it is possible to clearly identify what genes are present in each bacterium and perhaps gain insights into the ways by which the organisms have acquired the resistance genes, the extent to which these traits are the same in bacteria isolates from people, animals wastes and environmental media, and the possible sources or pathways of spread of these bacteria

Another area that should be further explored in future studies, is the potential for the presence and spread of antibiotic residues and residuals in the environment. It is known that in many people and animals, antibiotics are not fully metabolized within the body. Therefore, large quantities of the antibiotics and their active metabolites may be entering the environment. The rate of chemical or biological degradation of these drugs is once they reach the environment is uncertain. Furthermore, the concentrations of these drugs in environmental waters and soils are largely unknown. If these antibiotics are present in high enough concentrations in the environment, they may be creating selective pressure that increases the rate at which environmental bacteria acquire drug resistance. This in turn may result in increase risks to human exposed to these bacteria.

This research has made tangible contributions to our understanding the presence, sources and possible mechanisms of acquisition and transfer of antibiotic resistant enteric bacteria and the risks these bacteria may be posting to human health. However, this study leaves unanswered many questions about exposure sources and causality. Given the potential for serious public health risks from antibiotic resistant and pathogenic enteric bacteria, these unanswered research questions still need to be addressed in order to achieve the goal of obtaining conclusive answers.

Appendix A: Resistance pattern in Enterococcus sp. and E. coli

Table A1: Resistance patterns: Human E. coli

# of drugs	# of isolates	Resistance pattern *
3	1	FIS STR TET
	1	CHL FIS TET
	1	AMP NAL TET
	1	AMP SXT TET
	2	AMP STR TET
	3	FIS KAN TET
	3	AMP CIP NAL
4	1	FIS FOX GEN TET
	1	FIS KAN STR TET
	2	AMP FOX TET TIO
	2	AMP CHL FIS TET
	2	AMP FIS KAN TET
5	1	AMP CIP FIS GEN NAL
	1	AMP CHL CIP FIS TET
	1	AMP CHL FIS KAN TET
	2	AMP FIS KAN STR TET
	6	AMP FIS STR SXT TET
	6	AMP AUG FOX TET TIO
6	1	AMP CHL FIS STR SXT TET
	1	AMP CHL FIS NAL STR TET
	8	AMP CIP FIS GEN NAL SXT
7	1	AMP CHL FIS GEN NAL STR TET
	1	AMP CHL FIS NAL STR SXT TET
	1	AMP FIS FOX NAL STR SXT TET
	1	AMP AUG FIS FOX STR TET TIO
	2	AMP AUG FIS GEN NAL SXT TET
	9	AMP CIP FIS FOX GEN NAL STR
8	1	AMP AUG FIS FOX STR SXT TET TIO
	1	AMP AUG CIP FIS FOX GEN NAL STR
	2	AMP CIP FIS FOX GEN KAN NAL STR
	8	AMP CIP FIS GEN NAL STR SXT TET
9	6	AMP CIP FIS FOX GEN KAN NAL STR SXT
10	1	AMP CHL CIP FIS FOX GEN KAN NAL STR SXT
	1	AMP CIP FIS FOX GEN KAN NAL STR SXT TIO
	2	AMP CIP FIS FOX GEN KAN NAL STR SXT TET
	3	AMP AUG CIP FIS FOX GEN KAN NAL STR SXT

*Ampicillin (AMP), Quinupristin/Dalfopristin (AUG), Chloramphenicol (CHL), Ciprofloxacin (CIP), Sulfisoxazole (FIS), Cefoxitin (FOX), Gentamicin (GEN), Kanamycin (KAN), Naladixic Acid (NAL), Streptomycin (STR), Trimethoprim/Sulfamethoxazole (SXT) Ceftiofur (TIO), Tetracycline (TET)

Table A2: Resistance patterns: Environmental E. coli

# of drugs	# of isolates	Resistance pattern *
3	1	AMP CHL TET
	1	AMP NAL STR
	1	FIS SXT TET
	2	AMP FOX TET
	3	FIS STR TET
	4	AMP SXT TET
	5	CHL FIS TET
	5	FIS KAN TET
	6	AMP KAN TET
	7	AMP STR TET
	11	AMP FIS TET
4	1	AMP FIS STR TET
	1	FIS STR SXT TET
	1	AMP AUG FOX SXT
	1	AMP FOX TIO TET
	1	AMP AUG FOX SXT
	1	CHL FIS GEN TET
	1	AUG FIS SXT TET
	1	FIS KAN SXT TET
	1	AMP AUG TIO TET
	1	AMP KAN STR TET
	2	AMP CHL KAN TET
	2	AMP STR SXT TET
	2	AMP CHL FIS TET
	3	AMP FIS SXT TET
	3	FIS KAN STR TET
	4	CHL FIS KAN TET
	4	AMP FIS KAN TET
5	1	AMP FIS CHL STR TET
	1	AMP FIS STR SXT TET
	1	AMP AUG FIS SXT TET
	1	CHL FIS KAN SXT TET
	1	CHL FIS KAN STR TET
	1	AMP CHL FOX TIO TET
	1	AMP AUG FOX STR TET
	2	AMP CHL FIS KAN TET
	2	AMP FIS KAN STR TET
	2	AMP AUG FIS FOX TET
	2	AMP AUG FOX TIO TET
	6	AMP FIS KAN STR TET
6	1	AMP CHL FIS NAL STR TET
	1	AMP CHL FIS KAN SXT TET

	1	AMP FIS KAN NAL TIO TET
7	1	AMP CHL FIS GEN KAN STR TET
	1	AMP AUG FIS FOX STR SXT TET
	1	AMP AUG FIS FOX KAN TIO TET
	1	AMP AUG FIS FOX KAN STR TET
8	1	AMP AUG FIS FOX STR SXT TIO TET
	2	AMP AUG FIS FOX KAN STR TIO TET
9	1	AMP AUG CHL FIS FOX KAN STR TIO TET
	1	AMP AUG FIS FOX KAN STR SXT TIO TET

Table A3: Resistance patterns: Human Enterococci

# of drugs	# of isolates	Resistance pattern *
3	1	STR SYN TET
	1	DAP SYN TET
	1	DAP FLV TET
	1	FLV TGC TET
	1	FLV PEN TET
	1	CHL FLV TET
	4	ERY FLV TET
	10	FLV LIN TET
	71	LIN SYN TET
4	1	CIP FLV TET VAN
	1	LIN SYN TET TYLT
	1	FLV LIN PEN SYN
	1	DAP FLV LIN TET
	2	FLV LIN TET TGC
	2	CIP FLV LIN TET
	2	FLV LIN PEN TET
	3	LIN SYN TET TGC
	8	FLV LIN SYN TET
5	1	DAP FLV LIN SYN TET
	1	ERY FLV LIN PEN TET
	1	DAP ERYLIN TET TYLT
	1	ERY FLV LIN TET TYLT
	1	RYV FLV LIN SYN TET
	2	CHL ERY LIN SYN TYLT
	27	ERY LIN SYN TET TYLT
6	1	CHL ERY LIN SYN TET TYLT
	1	ERY GEN LIN STR SYN TET
	1	ERY LIN SYN TET TGC TYLT
	2	ERY GEN LIN SYN TET TYLT
	3	ERY FLV LIN SYN TET TYLT
	3	ERY FLV LIN STR TET TYLT
	6	ERY LIN STR SYN TET TYLT
7	1	DAP ERY FLV LIN STR SYN TET
	1	ERY FLV LIN PEN STR TET TYLT
	2	CHL ERY LIN STR SYN TET TYLT
	2	ERY FLV LIN STR SYN TET TYLT
	2	CHL ERY GEN LIN SYN TET TYLT
	3	ERY FLV GEN LIN STR TET TYLT
	4	ERY GEN LIN STR SYN TET TYLT
8	1	ERY FLV LIN PEN STR SYN TET TYLT
	2	CHL ERY GEN LIN STR SYN TET TYLT
	3	ERY FLV GEN LIN STR SYN TET TYLT

9	1	CIP ERY FLV GEN LIN STR SYN TET TYLT

*Chloramphenicol (CHL), Ciprofloxacin (CIP), Daptomycin (DAP), Erythromycin (ERY), Flavomycin (FLV), Gentamicin (GEN), Lincomycin (LIN), Penicillin (PEN), Streptomycin (STR), Quinupristin/Dalfopristin (SYN), Tetracylcine (TET), Tigecycline (TGC), Tylosin Tartrate (TYLT)

Table A4: Resistance patterns: Environmental Enterococci

# of drugs	# of isolates	Resistance pattern *
3	1	FLV LIN TYLT
	1	DAP LIN TET
	1	ERY LIN TET
	1	STR SYN TET
	1	LIN SYN TYLT
	2	CIP LIN SYN
	2	FLV SIN SYN
	2	DAP LIN SYN
	2	FLV LIN TGC
	4	LIN SYN TGC
	14	LIN SYN TET
	16	FLV LIN TET
4	1	ERY LIN SYN TET
	1	ERY FLV LIN SYN
	1	CIP LIN SYN TET
	1	GEN LIN SYN TET
	1	CHL LIN SYN TET
	1	LIN SYN TET TYLT
	1	ERY FLV LIN TET
	1	FLV LIN PEN TET
	1	CIP FLC LIN TET
	1	FLV LIN TET TGC
	1	ERY FLV LIN SYN
	1	FLV LIN STR SYN
	1	FLV LIN SYN TYLT
	2	LIN SYN TET TGC
	3	FLV LIN STR TET
	4	FLV LIN SYN TET
	8	LIN STR SYN TET
5	1	CHL ERY FLV LIN TYLT
	1	ERY FLV GEN LIN SYN
	1	FLV LIN STR SYN TET
	2	FLV LIN SYN TET TYLT
	3	LIN STR SYN TET TYLT
	17	ERY FLV LIN TET TYLT
	32	ERY LIN SYN TET TYLT
6	1	CHL ERY FLV LIN TET TYLT
	1	ERY FLV LIN TET TGC TYLT
	1	CIP ERY LIN SYN TET TYLT
	1	ERYGEN LIN SYN TET TYLT
	1	ERY GEN LIN STR SYN TYLT
	1	ERY FLV LIN STR SYN TET

	1	FLV LIN STR SYN TET TYLT
	1	DAP FLV LIN STR SYN TET
	2	DAP FLV LIN STR TET TYLT
	2	DAP ERY FLV LIN TET TYLT
	4	ERY LIN SYN TET TGC TYLT
	7	CHL ERY LIN SYN TET TYLT
	7	ERY FLV LIN STR TET TYLT
	19	ERY FLV LIN SYN TET TYLT
	26	ERY LIN STR SYN TET TYLT
7	1	CHL ERY FLV LIN SYN TET TYLT
	1	ERY FLV LIN SYN TET TGC TYLT
	1	ERY FLV LIN STR TET TCG TYLT
	1	CHLERY LIN STR SYN TET TYLT
	1	CHL DAP ERY LIN SYN TET TYLT
	1	CHL ERY FLV LIN STR SYN TYLT
	1	CHL ERY FLV LIN STR TET TGC
	2	ERY FLV GEN LIN SYN TET TYLT
	2	ERY FLV LIN PEN SYN TET TYLT
	2	DAP ERY LIN STR SYN TET TYLT
	3	ERY LIN STR SYN TET TGC TYLT
	12	ERY GEN LIN STR SYN TET TYLT
	21	ERY FLV LIN STR SYN TET TYLT
8	1	CIP ERY FLV LIN STR SYN TET TYLT
	1	CHL ERY FLV GEN LIN SYN TET TYLT
	1	CHL ERY FLV LIN PEN STR TET TYLT
	1	ERY GEN LIN PEN STR SYN TET TYLT
	1	ERY GEN LIN STR SYN TET TGC TYLT
	1	DAP ERY GEN LIN STR SYN TET TYLT
	1	ERY FLV GEN LIN STR SYN TET TYLT
	1	CHL ERY LIN STR SYN TET TGC TYLT
	1	CHL ERY FLV LIN STR SYN TET TYLT
	3	ERY FLV GEN PEN LIN SYN TET TYLT
	7	CHL ERY GEN LIN STR SYN TET TYLT
9	1	CHL ERY FLV GEN LIN STR SYN TET TYLT
	1	CHL ERY FLV LIN PEN STR SYN TET TYLT
	2	ERY FLV GEN LIN PEN STR SYN TET TYLT
10	1	CIP DAP ERY FLV GEN LIN STR SYN TET TYLT

Appendix B: Initial Questionnaire for Enrolled Participants

Evaluation of the Impact of Agricultural Operations on Water Quality in North Carolina

Interviewer initials:_____ Date of interview:_____

Place study id label here

Questionnaire

Your answers to this survey are voluntary and will be handled in a confidential manner. You may decline to answer any question.

Birth date____/____/____ Sex (circle one): Male Female

Home Address: _____

City: _____ State_____ Zip code:_____

County of residence (specify):_____

Ethnicity (circle one): Caucasian/white African American/Black Hispanic
Asian Native American

Country of birth: _____

If you were not born in the United States when did you come to the U.S.? Month_____ Year_____

Work place and occupation

1. What is your current occupation (type of job)? Please check one.

_____Farmer/laborer working with animals
_____Farmer/laborer not working with animals
_____Slaughterhouse worker
_____Meat Packer
_____Truck driver
_____Maintenance/repair
_____Construction worker
_____ Feed plant laborer
_____ Factory worker
_____ Clerk/clerical/administrative (desk job)
_____ Store clerk/retail
_____ Nurse, nurse assistant, medical worker
_____ Other (specify)_____

2. Current Place of Work (put a number 1 by your main place of work and number 2 by your next most important place of work if you have one)

_____ Cattle/Dairy (cow) farm
_____ Poultry (chicken or turkey) farm
_____ Swine (pig) farm
_____ Farm without animals
(example: tobacco farm)
_____ Trucking company for farm animals
_____ Trucking company for animal feed
_____ Trucking company for merchandise
_____ Slaughterhouse
_____ Meat packing company
_____ Construction company
_____ Animal feed company
_____ Manufacturing company
_____ Store
_____ Hospital or medical clinic
_____ Other (specify)_____

3. Have you worked at your current job for less than 1 year? YES NO

If yes, where did you previously work? (put a number 1 by your main place of work and number 2 by your next most important place of work if you have one)

_____ Cattle/Dairy (cow) farm
_____ Poultry (chicken or turkey) farm
_____ Swine (pig) farm
_____ Farm without animals
(example: tobacco farm)
_____ Trucking company for farm animals
_____ Trucking company for animal feed
_____ Trucking company for merchandise
_____ Slaughterhouse
_____ Meat packing company
_____ Construction company
_____ Animal feed company
_____ Manufacturing company
_____ Store
_____ Hospital or medical clinic
_____ Other (specify)_____

4. If you have been in your current occupation for less than 1 year, what was your former occupation (type of job)? Please check one.

_____Farmer/laborer working with animals
_____Farmer/laborer not working with animals
_____Slaughterhouse worker
_____Meat Packer
_____Truck driver
_____Maintenance/repair
_____Construction worker
_____ Feed plant laborer
_____ Factory worker
_____ Clerk/clerical/administrative (desk job)
_____ Store clerk/retail
_____ Nurse, nurse assistant, medical worker
_____ Other (specify)_____

5. What town/county is your occupation located in? _____

6. Please check your total family yearly income before taxes. This includes the combined money from jobs, net income from business, farm or rent, pensions, dividends, interest, social security payments and any other money received by members of your family who are 15 years of age or older (please check only one).

- ❑ Less than $5,000
- ❑ 5,000 to 7,499
- ❑ 7,500 to 9,999
- ❑ 10,000 to 12,499
- ❑ 12,500 to 14,999
- ❑ 15,000 to 19,999
- ❑ 20,000 to 24,999
- ❑ 25,000 to 29,999
- ❑ 30,000 to 34,999
- ❑ 35,000 to 39,999
- ❑ 40,000 to 49,999
- ❑ 50,000 to 59,999
- ❑ 60,000 to 74,999
- ❑ $75,000 or more

Animal Contact

7. Do you have any pets? YES NO

7a. If YES, which pets (circle all that apply):

Dogs Cats

Birds Reptiles/snakes Other _____

7b. Have you had frequent contact with your pets in the last 30 days? YES NO

7c. Have any of your pets been sick with vomiting or diarrhea in the past 30 days? YES NO

7d. If YES above, which pet was ill? _____

8. Have you come into contact with or handled any animal waste or manure in the past 30 days? YES NO

9. Do you have contact with farm animals or animal products in your current job? YES NO

If YES, please circle how often you have contact with each animal
(Use the last 30 days to answer this question)

9a. Cattle: Many times per day Once or twice per day Once or twice per week
Once or twice per month Less than once a month Never

9b. Chicken: Many times per day Once or twice per day Once or twice per week
Once or twice per month Less than once a month Never

9c. Swine: Many times per day Once or twice per day Once or twice per week
Once or twice per month Less than once a month Never

8d. Turkey: Many times per day Once or twice per day Once or twice per week
Once or twice per month Less than once a month Never

8e. Other: Many times per day Once or twice per day Once or twice per week
Once or twice per month Less than once a month Never

8f. Have any of the animals been sick with vomiting or diarrhea in the past 30 days? YES NO

10. If you have been in your current job for less than one year did you have contact with farm animals in your previous job? YES NO

Antibiotics and Illness

11. Have you been treated by a doctor in the last 30 days? YES NO

11a. If YES

When? _____ What for? _____
When? _____ What for? _____

12. Have you taken any antibiotics during the last 30 days? YES NO

12a. If YES, list antibiotics and the conditions they were prescribed for (if you can't remember th exact antibiotic name, do your best and spell out as much as you can):

medication: _____ for _____
_____ for _____
_____ for _____
_____ for _____

13. Have you stayed in the hospital overnight or longer in the last year? YES NO

13a. If YES, approximately when were you in the hospital?

___/___/___ to ___/___/___

13b. What was the reason for your stay in the hospital? _____

14. Have you had diarrhea in the past 2 weeks? YES NO

15. Do you have diarrhea today? YES NO

If yes, and it has lasted more than 48 hours, please check all symptoms that apply below.

- ❑ Fever
- ❑ Mucus present in stools
- ❑ Blood present in stools
- ❑ Cramping or other abdominal (stomach) pain

16. Do you feel well today? (circle one) YES NO

16a. If NO, describe how you are feeling _____

17. Have you taken prednisone, cortisone or other or steroids in the past 6 months? YES NO

18. Do you regularly take antacids or medicine to lower stomach acid? YES NO

19. Do you have a history of any of the following chronic illnesses? (Please check all that apply):

a. Diabetes
b. Kidney/renal disease
c. Liver disease
d. Stomach or intestinal diseases
e. Lung/pulmonary disease
f. Immune system disease or HIV infection
g. Cancer or leukemia

20. If you work with animals, have any been given antibiotics to treat an illness in the last 30 days?

YES NO

If yes, please list the antibiotics (if you can't remember the exact antibiotic name, do your best and spell out as much as you can): _____

Diet

21. Do you eat raw or very rare beef, pork or chicken? YES NO

22. Do you eat soft Mexican cheese or other unpasteurized products? YES NO

Water Use

23. What is the source of your drinking water? (circle one)

Private well Community/city water supply

Other_____

23a. If Private well, has the water quality of the well been tested? YES NO

23b. If YES, when (approximate date)? ____/____/____

What were the results of the testing? _____

23c. If Private well, have you had to put chlorine bleach in your well in the last 30 days? YES NO

24. Do you work in any body of water? YES NO

24a. If YES, which types (circle or list all):

Lakes Ponds Rivers

Streams Drainage ditches Oceans

Estuaries (i.e., where salt water from the ocean meets fresh water from a river)

Other _____

25. Do you use any body of water for recreational purposes? YES NO

25a. If YES, which types (circle or list all):

Lakes Ponds Rivers

Streams Drainage ditches Oceans

Estuaries (i.e., where salt water from the ocean meets fresh water from a river)

Other _____

Waste Disposal

26. How do you dispose of your household sewage? (circle one)

City or county sewer Septic tank

Other (specify) _____

Travel Information

27. Have you traveled to any countries outside of the United States? YES NO

27a. If YES, which country/countries did you travel to?

_____ _____

27b. When was the last time you were in each of the countries above (approximately)?

Date ____/____/____ ____/____/____

27c. Were you ill with vomiting and/or diarrhea during any of the above trips? YES NO

27d. Did you take any new medications while traveling? YES NO

27e. What new medications did you take?_____

_____

Thank you for your help with our project.

Appendix C: Monthly Questionnaire to accompany specimens

MONTHLY QUESTIONNAIRE

Evaluation of the Impact of Flooding on Water Quality and Human Health Indicators in North Carolina
North Carolina Division of Public Health
Wake Forest University School of Medicine, Department of Medicine

1. Has your occupation changed? (circle one) YES NO

   1a. If YES, what is your new occupation? _____

2. Have you been treated by a doctor in the last 30 days? (circle one) YES NO

   2a. If YES When? _____ What for? _____
   When? _____ What for? _____

3. Have you taken any medications during the last 30 days? (circle one) YES NO

   3a. If YES, list medications and the conditions they were prescribed for:
   medication: _____ for _____
   medication: _____ for _____
   medication: _____ for _____
   medication: _____ for _____

4. Have you stayed overnight in the hospital in the last 30 days? (circle one) YES NO

   4a. If YES, approximately when were you in the hospital? ____/____/____ to __/____/____

   4b. What was the reason for your stay in the hospital? _____

5. Have you had diarrhea (defined as at least 3 watery stools per day over a 2 day period) in the last 30 days? (circle one) YES NO

6. Do you feel well today? (circle one) YES NO

   6a. If NO, describe how you are feeling_____

Thank you for your help with our project.

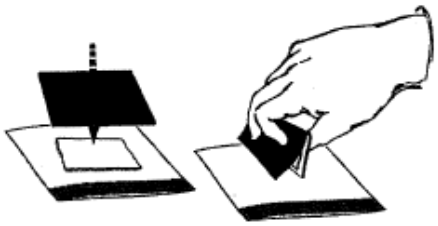
Appendix D: Human Fecal Sample Submission Instructions

INSTRUCTIONS FOR STOOL SAMPLE COLLECTION

1. Just **BEFORE** you have a bowel movement:
   - Take one sponge (moistened with transport medium) out of its pouch
   - On a flat surface within reach, lay sponge on top of the pouch

2. Immediately **AFTER** you have a bowl movement:
   - Take one orange pad out of the bag
   - Use the white, soft side to wipe anal area

3. Put the orange pad – white side down – on top of the sponge.
   Fold both pads in half together so the plastic orange backing is on the outside.



4. Open the pouch the sponge came from.
   Put the two folded pads into the pouch together and seal firmly.

5. Fill out the monthly questionnaire and put it in one of the return envelopes provided along with the sealed pouch and mail to our laboratory.

Specimens should be collected on a **Sunday** or **Monday** and shipped either **Monday** or **Tuesday** to our laboratory.

If you have any questions, please call Victor Varela at **(877) 655-0433** (toll free) weekdays or **(336) 978-4966** on weekends and evenings.

Appendix E: Antibiotics used by site for therapeutic purposes or in feed to maintain health and growth in the herd

Antibiotic	SITE										
	1	2	3	4	5	6	7	9	10	11	12
Tetracycline	X				X	X					X*
Penicillin	X				X	X					X
Tulathromycin (macrolide)											X
Ampicillin											X
Sulfa drugs	X				X	X					

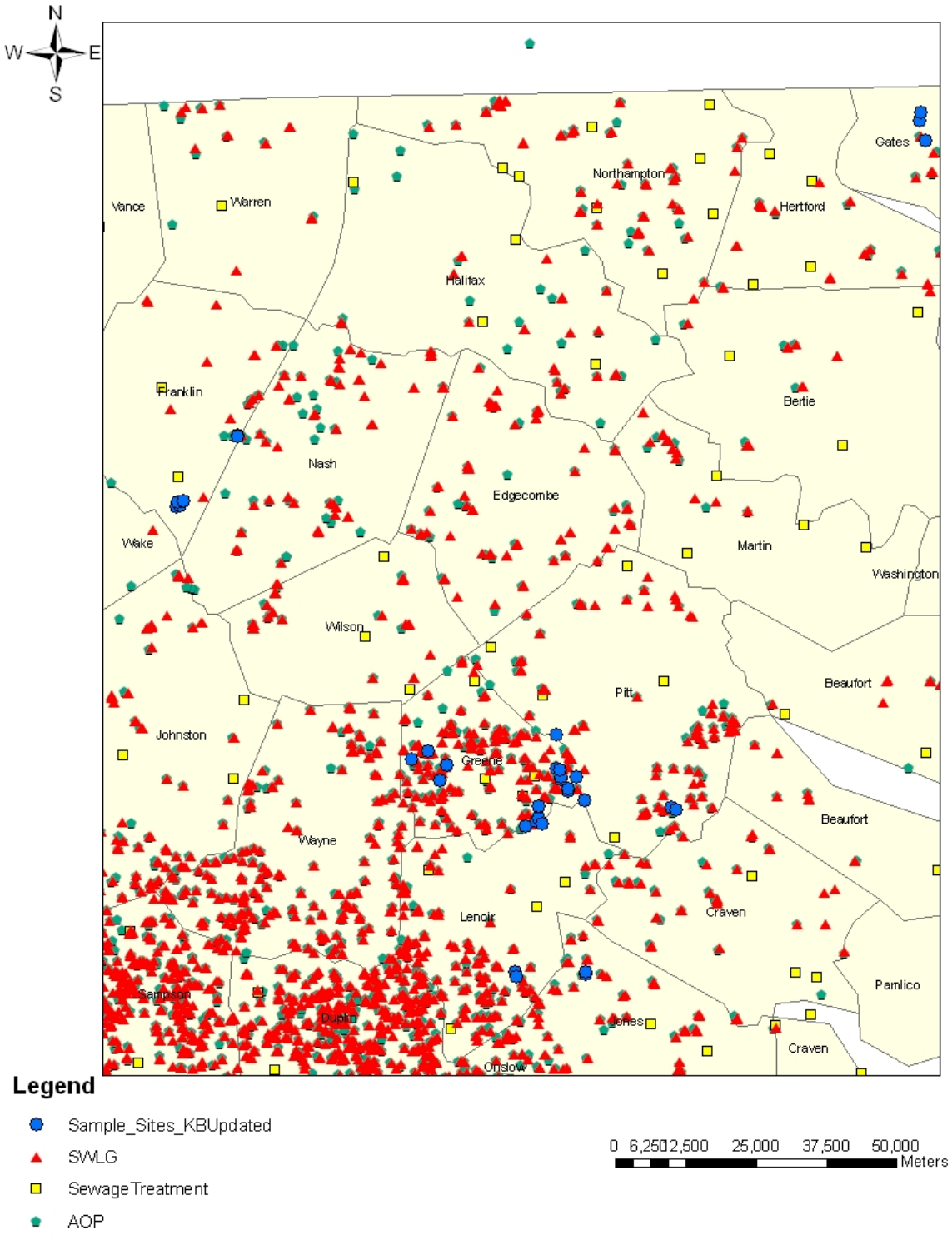
X used therapeutically

*used in feed

Appendix F: Antimicrobial Classes of Antibiotics Used in Phenotypic Profiling

Antimicrobial Class	Subclass (if any)	Drug Used	Plate Using (gram+/gram-)
Penicillins	Penicillin (natural)	Penicillin	Gram Positive
Penicillins	aminopenicillins	Ampicillin	Gram Negative
Penicillins*	B-lactase/β-lactamase inhibitor combo	Amoxicillin/ Clavulanic Acid	Gram Negative
Cephems	Cephamycin	Cefoxitin	Gram Negative
Cephems	Cephalosporin II	Ceftriaxone	Gram Negative
Cephems	cephalosporin	Ceftiofur	Gram Negative
Aminoglycosides		Amikacin	Gram Negative
Aminoglycosides		Gentamicin	Both
Aminoglycosides		Kanamycin	Both
Aminoglycosides		Streptomycin	Both
Quinolones		Naladixic Acid	Gram Negative
Fluoroquinolones		Ciprofloxacin	Both
Folate Pathway Inhibitors		Sulfisoxazole	Gram Negative
Folate Pathway Inhibitors		Trimethoprim/ Sulfamethoxazole	Gram Negative
Lipopeptides		Daptomycin	Gram Positive
Phosphoglycolipid		Flavomycin	Gram Positive
Macrolide		Erythromycin	Gram Positive
Macrolide		Tylosin Tartrate	Gram Positive
Nitrofurans		Nitrofuratoin	Gram Positive
Oxazolidinines		Linezolid	Gram Positive
Glycopeptides	glycopeptides	Vancomycin	Gram Positive
Phenicols		Chloramphenicol	Both
Streptogramins		Quinupristin/ Dalphopristin (Augmentin™)	Gram Negative
Tetracycline		Tetracycline	Both
Glycylcycline		Tigecycline	Gram Positive
Lincosamide		Lincomycin	Gram Positive

Appendix G: Map of Surface Water Sampling Sites (blue) with Swine Lagoons (red), Animal Operation Permits (green) and Sewage Treatment Plants (yellow)



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